Efficient Protoplast Isolation Towards an Adapted Culture Protocol for Cotton (*Gossypium* sp.)

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ABSTRACT

The media composition for successful cotton (Gossypium sp.) protoplast isolation and cultivation was determined through systematic investigation of all the components of each culture medium. A set of orthogonal design experiments was used to verify the effect of each compound of the initial medium alone and in combination with other compounds. This allowed defining the optimum composition of the media needed to successfully cultivate protoplasts through different culture stages.

Introduction

Protoplast culture has been used in many species for developing fast, reliable procedures for induction of somatic embryogenesis. The technique was also applied to cotton (Finer and Smith, 1982; Firoozabady and DeBoer, 1986), but success was reported only when established embryogenic cultures were used as a protoplast source (Peeters *et al.*, 1994; She *et al.*, 1995). Attempts to use other explants for protoplast isolation failed because of poor plating efficiency. Assuming that cotton cells have specific requirements for growth *in vitro*, we chose to re-evaluate the overall composition of the media with protoplasts and young colonies.

In previous work with tree protoplasts (Chupeau *et al.*, 1993), the mode of preparation of protoplasts was shown to be crucial for their ultimate development. Therefore, the effect of various conditions of maceration on the capacity of protoplasts to survive and divide was systematically explored. Special attention was then paid to the role of the chemical form and concentration of nitrogen and auxin sources before reviewing the effects of other salts and organic nutrients. This paper reports the development of an efficient protocol for isolation and culture of protoplasts from three cotton genotypes.

Material and Methods

Plant material. Seeds were surface-sterilized with a commercial bleach solution and germinated on basic medium (Table 1). Young plantlets were obtained under standard culture room conditions of 26-28°C, 70-90% relative humidity and 16 hour light period. Plantlets from two Bulgarian cotton varieties (Chirpan 432 and Chirpan 603) and Coker 310 were transferred to pots and cultivated in a growth chamber where the same humidity and photoperiod were maintained, but the temperatures were 28±1°C during the daytime and 15±1°C during the night. These plants were used as sources of true leaves for protoplast isolation. The studies were made using the two Bulgarian varieties and the best treatments were verified on Coker 310.

Protoplast isolation. Two types of explants were used for protoplast isolation: hypocotyls from 14 to 21-day-old, *in vitro* grown seedlings, and 10-14-day-old true leaves. The initial ionic composition of the protoplast isolation medium was as described previously (Chupeau *et al.*, 1993). Three maceration enzymes (Cellulase Onozuka R10, Drisellase, and Macerozyme R10) were used for cell wall digestion. Various combinations were tested to establish the best digestion mixture.

Released protoplasts were separated from debris with 80 µm sterile metal sieves and washed twice through centrifugation in osmotic saline buffer (330 mM KCl, 14 mM $CaCl_2 \cdot 2H_2O$, 3.5 mM MES, 8 mg/L BCP, pH 5.5). After the second wash protoplasts were plated at a final concentration of $5x10^4$ protoplasts/mL.

Culture of protoplasts. Low ionic strength medium (medium M1, Table 1) was the basic solution to which inorganic salts under investigation were added at different concentrations. Also tested were the effects of 2,4-D, 2,4,5-T, IAA, NAA, TDZ, BA and kinetin in rectangular-design experiments. Each auxin was tested against each cytokinin in at least three levels.

Division rates and plating efficiency (number of micro-colonies obtained) were calculated as percent of initially plated protoplasts.

Proceedings of the World Cotton Research Conference-2. Athens, Greece, September 6-12, 1998. pp.330-334.

Results

The approach of Chupeau *et al.* (1993) was used to optimize conditions for protoplast cultivation. Optimizing the composition of the medium by studying its constituents through their stepwise introduction resulted in a reduced number of treatments and made the variation of all of the compounds possible.

Protoplast isolation

Toxicity of Macerozyme and requirement for Ca^{2+} in digestion mixture. In preliminary studies with different explants (hypocotyl, cotyledon, callus and embryogenic suspensions) hypocotyl segments appeared to yield the most uniform protoplasts with best viability. In this study hypocotyl segments were compared to true leaves as explant sources. Under described conditions protoplasts from both types of explant had smooth surfaces and even distribution of chloroplasts (Fig. 1). Nevertheless, at the early stages of this study the protoplasts could not be cultivated for more than 2-3 days. Even when plated at low levels of all the macroelements to avoid possible toxicity, most of the protoplasts would collapse within 4 days of cultivation. Therefore, toxic effect of some of the maceration enzymes was suspected. To verify this assumption a set of experiments was designed to test combinations of the enzymes on yields and subsequent development.

Cellulase below 0.5% was ineffective for protoplast release (less than $1x10^5$ pp/g). Drisellase or Macerozyme alone did not release sufficient number of protoplasts either. The highest yields from hypocotyl explants ($4x10^6$ pp/g) were obtained with 1% Cellulase + 0.01% Macerozyme + 0.02% Drisellase. The most effective combination when true leaves were used as an explant was 0.75% Cellulase with 0.2% Drisellase, resulting in $3.6x10^8$ protoplasts per gram explant. However, none of the protoplasts from isolations where Macerozyme was used could be successfully cultivated. Protoplasts underwent further development and began dividing only when digestion media contained no Macerozyme. Subsequent isolations were with 1% Cellulase + 0.02% Drisellase for hypocotyl explants (resulting in an average yield of $1.5x10^6$ pp/g) and 0.75% Cellulase + 0.2% Drisellase for true leaves.

Culture of protoplasts

Nitrogen toxicity. In spite of good protoplast yields and viability at the time of isolation, high mortality (up to 98%) was observed three days after isolation (DAI). This observation suggested possible toxicity of some of the cultivation medium compounds to the wall-deprived cells. Calcium and nitrogen were considered the first inorganic compounds for which exact concentrations should be determined. A rectangular design, where the range of $CaCl_2$ (0 -10 mM) was tested against 0 - 8 mM NH_4NO_3, was chosen for the first assay. Toxic levels of NH_4NO_3 were reached at very low concentrations (2 mM) (Fig. 2a), but $CaCl_2$ did not result in immediate apparent harm to the protoplasts. Depriving the cultures of calcium led to some enlargement of the cells 3-5 days after plating (Fig. 2b).

Essential role of magnesium. Considering the toxic effects of nitrogen, a range below the 2mM level (0, 0.5, 1 and 1.5 mM) was tested against 0-10 mM $MgSO_4$. The medium was supplemented with 2 mM $CaCl_2$, observed to be sufficient for preventing increase of cell size. Absence of $MgSO_4$ resulted in shrinkage of protoplasts 2 DAI (Fig. 2c). The difference in response to the two higher levels was not distinguishable during the first week of cultivation. The longest cultivation periods were achieved when the medium was supplemented with 1.5 mM $MgSO_4$ and 1 mM NH_4NO_3.

High levels of 2,4,5-T and TDZ are needed for initiation of divisions. The improved ionic composition of the cultivation medium was used to determine the hormone combination that could stimulate the protoplasts into dedifferentiated dividing cells. The auxins 2,4-D at 0.05, 0.25 and 0.5 µM and 2,4,5-T at 4, 10 and 40 µM were tested individually against TDZ at 1, 3 and 7 µM. Both auxins induced division of protoplasts, but the lowest effective dose for 2,4-D was 0.25 µM, while at least 10 µM 2,4,5-T was required to obtain divisions. Similar to 2,4,5-T, high levels of TDZ appeared essential to induce divisions. Rare divisions were obtained at 1 µM, but the rates increased with increasing TDZ concentration and reached their highest levels (20-30% of initially plated protoplasts) in the treatments where TDZ was added at 7 µM.

Ca^{2+} blocks the division of protoplasts. The optimal hormone regime for division initiation was considered established from these experiments. Nevertheless, higher division rates were desirable to provide sufficient micro-colonies for the further experiments so attempts to improve the mineral composition of the media were resumed.

The effects of Ca, N and Mg were verified, as described above. Concentrations of 1 mM NH_4NO_3, 4 mM $CaCl_2$ and 2 mM $MgSO_4$ resulted in the longest periods of protoplast cultivation. However, presence of $CaCl_2$ in the plating media retarded (at 2 or 4 mM) or even blocked divisions (at 8 mM). Only when media were completely devoid of calcium were high division rates obtained (60-70% of initially plated protoplasts) early in the cultivation (4 DAI) (Fig. 3).

Discussion

The experiments on protoplast isolation revealed several points that require particular attention to obtain healthy cotton protoplasts that can continue further development. One unexpected observation was the toxicity of Macerozyme to hypocotyl and leaf protoplasts. This was in contrast to a number of

previous studies where no such effect was observed (Finer and Smith, 1982; Firoozabady and DeBoer, 1986; Peeters *et al.*, 1994). There could be at least two reasons for the difference. In most of the studies, where success was reported, callus-derived protoplasts were used. Cells that have been cultivated *in vitro* for a long time are known to have pronounced changes in their biochemistry. On the other hand, the toxicity of the Macerozyme does not affect the fluorescence of the widely used viability tester fluorescein diacetate (FDA). This could explain why, in spite of the good viability observed by FDA tests, poor first division rates and plating efficiencies were obtained in studies where Macerozyme was used as a digestion enzyme.

The form in which nitrogen is applied may be crucial for the induction of somatic embryogenesis in cotton (Trolinder and Goodin, 1988). Contrary to their observations, KNO_3 did not have well pronounced effect on the cultures. In contrast, protoplasts were very sensitive to the time and concentration of NH_4NO_3. Even 1.5 mM was sufficient to decrease significantly the viability of the protoplasts. These effects could be explained if the presence of NH_4^+ in the medium is the cause of the toxicity, not the NO_3^-. In the initial stages of cultivation, sufficient nitrogen, preserved from the cells of origin, is present in the protoplasts. Before the first division NH_4^+ could not be metabolized and rapidly reached toxic levels in the cell. After the second division, the cells could efficiently use the NH_4^+ ions. The same type of response was observed with protoplasts from other woody species. It confirms that such a response is a general rule and the same nitrogen supply pattern should be followed for other species.

Sensitivity of cotton to magnesium deprivation is well established in field experiments by the phenomenon known as “red leaf” disease. Shrinking of isolated protoplasts complements the whole plant response, providing additional evidence for the importance of this macroelement.

In the present study, the need for high hormone levels to induce division in protoplast cultures is well established. Our experiments confirmed that the efficient conversion of cells, present in the protoplast culture, into rapidly dividing dedifferentiated cells requires elevated hormonal levels.

The most striking observation in this study was the peculiar path of Ca^{2+} supply that has to be followed to cultivate successfully cotton protoplasts. As an important component in many cell compartments and signal transduction pathways, the higher levels of Ca^{2+} promote better cell development. The observation that low levels of this element are required for induction of division was surprising. When elevated levels of $CaCl_2$ were applied, apparent thickening of the cell wall was observed. Therefore, we concluded that calcium application results in cell wall stabilization, thus reducing the permeability. Most probably, this effect is combined with the response to the growth regulators, which is calcium dependent as well. These results demonstrate that improving the nutritional conditions for the cotton protoplasts results in significantly improved division rates and plating efficiencies. Further studies will be needed to determine the best media compositions that will be needed for the development of a successful cultivation-regeneration protocol.

Acknowledgement

The present study was supported by a NATO grant for Bojin Bojinov.

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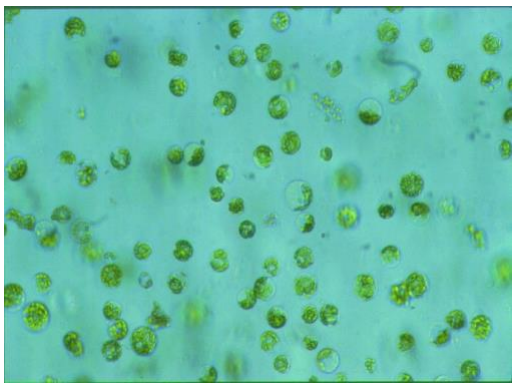
Table 1. Composition of media used for cotton protoplast culture.

Components	Basic	M1	M2	M3
Macroelements - mg/l				
KNO_3	150	150	150	150
NH_4NO_3	80	-	80	640
$CaCl_2$	110	-	-	110
$MgSO_4.7H_2O$	180	-	360	540
KH_2PO_4	20	20	20	80
Microelements - mg/l				
Fe cit. NH_4	50	50	50	50
KJ	0.01	0.01	0.01	0.01
H_3BO_3	1	1	1	1
$MnCl_2.4H_2O$	-	-	-	-
$MnSO_4.4H_2O$	0.1	0.1	0.1	0.1
$ZnSO_4.7H_2O$	1	1	1	1
$Na_2MoO_4.2H_2O$	0.9	0.9	0.9	0.9
$CuSO_4.5H_2O$	0.03	0.03	0.03	0.03
$CoCl_2.6H_2O$	0.03	0.03	0.03	0.03
$AlCl_3$	0.03	0.03	0.03	0.03
$NiCl_2.6H_2O$	0.03	0.03	0.03	0.03
Vitamins - mg/L				
Inositol	100	100	100	100
Pantothenate Ca	1	1	1	1
Biotin	0.01	0.01	0.01	0.01
Niacin	1	1	1	1
Pyridoxine	1	1	1	1
Thiamine	1	1	1	1
Additives				
2,4,5-T µM	-	-	40	20
TDZ µM	-	-	10	5
MES g/L	0.7	0.7	0.7	0.7
BCP mg/L	8	8	8	8
Glucose g/L	10	20	20	20
Mannitol g/L	-	90	90	90
Glutamine mg/L	146	146	146	146

Figure 1. Protoplasts isolated from (a) true leaves (x240) and (b) hypocotyl explants (x400), showing good cell wall digestion, smooth protoplast surface and even distribution of chloroplasts.



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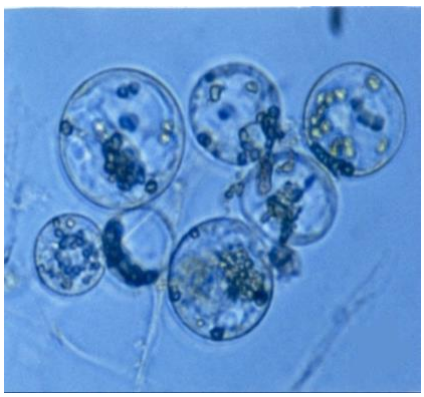
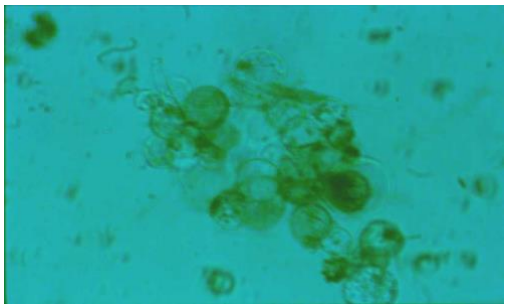


b

Figure 2. The effect of macroelements on the cultured protoplasts 4 days after isolation (all pictures x240): (a) 2 mM NH_4NO_3; (b) Calcium deprivation; (c) withdrawal of magnesium from the cultivation medium.



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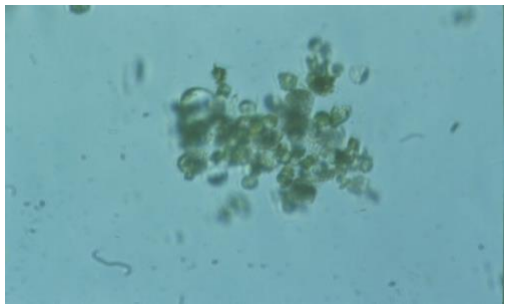

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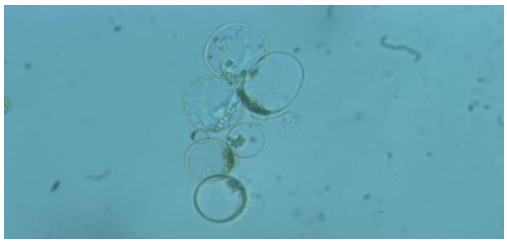

b.

Figure 3. Multiple divisions induced in the calcium-deprived medium, supplemented with 40 µM 2,4,5-T and 10 µM TDZ (x 240).