Cotton Protoplast Culture

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Introduction

Since World War II the predominant position of cotton as a textile fiber has been threatened by the development of synthetic fibers. In order for cotton to remain competitive, continuous fundamental and applied research is necessary to improve the quality of cotton fibers.

Intensive classical breeding efforts resulted in high yielding and high quality tetraploid cottons. Salt tolerance, insect and pest resistance, glanded plants with glandless seeds and climatological adaptations are objectives of many cotton breeding programs. Although a rich and useful genetic reservoir remains to be exploited in wild and wild relatives of cotton, further improvement through hybridization has become difficult because of incompatibility barriers.

These limitations resulted in alternative ways of genome improvement. Different techniques have been developed to transfer new genes into plants and the first transgenic cotton plants have been obtained. Detailed knowledge of the gene structure and regulation of its expression at the molecular level is necessary to introduce into the genome novel genes with interesting features. *Agrobacterium tumefaciens* is a vector system with the capacity for gene transfer to many plant species. This transformation technique has its limitations, however, because of its limited host range, low transformation efficiency, problems with bacterium removal and DNA manipulation in large plasmids. Alternative techniques for gene transfer such as biolistic or particle guns have created high expectations. Microparticles coated with DNA coding for a novel feature are accelerated and shot into cells or tissues. A detailed review on approaches and results of gene transfer into plants was recently published (Potrykus, 1991). The decisive step in the transformation process remains the regeneration of stable plants from the transformed cells.

Genetic engineering of cotton plants is currently the objective of many researchers. Fundamental information on the applicability of different techniques of gene transfer has been gathered and the first successful transformations have been achieved. Herbicide and insect tolerant transgenic cotton lines have been developed (Bayley et al., 1992; Perlak et al., 1990). Because of its superior regeneration ability, Coker 312 was used in these transformation experiments. Back-crossing is still required to incorporate the novel characteristics into commercial cultivars. An excellent updated review on achievements and perspectives in cotton biotechnology was recently reported by Stewart (1991) and published in the series of ICAC review articles.

Objectives of Protoplast Culture

The above mentioned transformation techniques used cells as a source material. However, protoplasts are recognized to be ideal for gene transfer (Potrykus, 1991) because the cell wall is removed (no longer a physical barrier) and thus the plasmalemma becomes freely accessible. The frequency that genes reach and enter a protoplast is enhanced. The DNA uptake has become a physical process. Since no biological vector is required, the host range problem is circumvented. Electroporation and microinjection are gene transfer techniques considered for protoplasts.

Genetic improvement can also be realized through fusion of protoplasts resulting in somatic hybrids. Chemical fusion using polyethylene glycol and electrofusion are the techniques mainly used for protoplast fusion. Protoplasts also create the possibility of recovering plants from a single cell origin (no chimera) and of selecting clones with novel characteristics via somaclonal variation. Until now, plant regeneration from cotton protoplasts is the limiting factor for the application of transformation techniques on protoplasts.

Protoplasts are also the material of choice for fundamental studies. Cell organelles and constituents can be better isolated from protoplasts than from tissues. The plasmalemma of *Daucus carota* protoplasts has been isolated and characterized (Boss and Ruesink, 1979). Intact chloroplasts, vacuoles, mitochondria and nuclei have been isolated from protoplasts by an osmotic shock and gradient centrifugation (Wagner and Siegelman, 1975; Ohyoma et al., 1977; Tallman and Reeck, 1980). Protoplasts are also used to study cell wall synthesis (Mock et al., 1990). Absence of the rigid wall facilitates the chemical and physical analysis of the cell membrane (Reinert and Bajaj, 1977; Stafford and Warren, 1991) and the study of specific cell wall enzymes (Fry, 1988). Protoplasts are also used in very specific research programs. The metabolism of C4 plants was studied using bundle sheaf protoplasts of C4 plants (Edwards and Huber, 1978). Light and stomatal functions were studied on guard cell protoplasts (Zieger and Hepler, 1979). Somatic cell genetics can also be studied with protoplasts (Binding, 1986). The regeneration capabilities of differentiated cells and the genetic basis for loss or preservation of regeneration ability in the course of cell differentiation can be analyzed.

Current Achievements on Cotton Protoplast Culture

In order to use protoplasts for genome improvement, protoplast technology needs to be developed. Protoplasts or plant cells

without a wall were originally obtained by mechanical isolation. Nowadays the protoplasts are obtained by enzymatic digestion of the wall using mainly cellulase and pectinase. Isolation is done in such a way that protoplasts are not damaged but retain their ability to synthesize a new wall, as well as divide and regenerate into intact and stable plants.

The first isolation and culture experiments with cotton protoplasts were reported by Bhojwani et al., 1977. Protoplasts were isolated from hypocotyl-derived callus of *G. hirsutum*. The protoplasts were cultured in liquid medium. The first divisions were observed after 6 days and resulted in the formation of colonies consisting of 25-30 cells maximum after 5 weeks.

Finer and Smith (1982) reported the culture of protoplasts from friable hypocotyl-derived callus of *G. klotzschianum*. Isolation efficiency was influenced by the callus age, incubation time in enzyme mixture, concentration of osmoticum and agitation speed during digestion.

Division was observed 3 days after isolation and multicellular colonies were formed after 2 weeks. The regeneration capability of protoplasts isolated from hypocotyl or young stem tissue seems to be more limited than the one reported for cotton callus protoplasts since cotton cotyledon-derived protoplasts formed microcolonies of only 2-3 cells in *G. hirsutum* and 5-8 cells in *G. barbadense* (Firoozobady and DeBoer, 1986). A high rate of cell wall regeneration and cell division of freshly isolated protoplasts is required for optimal plating efficiency and successful plant regeneration. Firoozobady (1986) demonstrated that the ability of cotyledon protoplasts to regenerate new cell wall and undergo division depends upon the stage of the cell cycle at the time of isolation, which is dependent upon the age and growth condition of the donor tissue.

Thomas and Katterman (1984) isolated protoplasts from callus obtained from anthers of *G. hirsutum*. The yield of viable protoplasts is greatly enhanced when protective agents are used in the enzyme mixture. Ca^{2+}, Mg^{2+} or certain amino acids prevented the spontaneous lysis of protoplasts in the presence of RNA contaminants in the cellulase enzyme preparation. Using these protection agents macroscopic callus was obtained after 3 weeks culture.

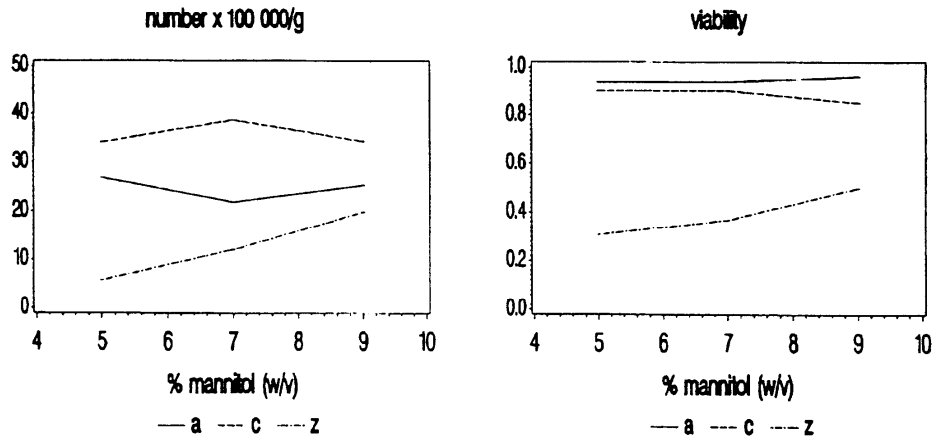
A method for the isolation and culture of protoplasts isolated from stem callus of *G. hirsutum* which leads to normally growing callus tissue was reported by Saka et al., (1987).

The highest regeneration stage obtained from cotton protoplasts is callus, and until now no reports have been presented on successful plant regeneration from cotton protoplasts.

In 1991 a research program, studying the genetic variability in cotton protoplast culture, was started at the Laboratory of Tropical Crop Husbandry with financial assistance from the Catholic University of Leuven (Belgium) and N.F.W.O. (Belgium). *G. hirsutum* cv. Coker 312 characterized by a high regeneration ability (Trolinder and Xhixian, 1989), *G. australe* a wild diploid and *G. hirsutum* cv. Zeta 4 a Greek commercial cultivar are being used in this study.

A fractionated factorial experiment was laid out. Factors recognized to be important in protoplast isolation were analyzed. These were osmotic conditions, incubation time, pH and enzyme concentrations. Yield and viability of cotyledon protoplasts of the 3 different varieties were assessed. Tendency diagrams demonstrate the variety-dependent influence of the considered factors. The influence of mannitol concentration on protoplast yield and viability is illustrated in Figure 1.

Figure 1: Influence of mannitol concentration on protoplast yield and viability (ratio of number viable protoplasts to total number) of *G. australe* (a) and *G. hirsutum* Coker 312 (c) and Zeta (z).



Results also indicated that protoplast characteristics such as size and chloroplast content depend upon isolation protocol. The average Coker 312 protoplast diameter of the different isolation experiments ranged between 20 and 27µm. The regeneration ability of these different protoplasts needs to be assessed.

Isolation conditions of cell suspensions and cotyledon-derived protoplasts of Coker 312 were investigated. Optimal conditions were different for both explants, suggesting that cell and wall constituents of cotyledon cells and cell suspensions are different. The influence of pH on protoplast yield and viability of both explants is illustrated in Figure 2.

Several reported techniques were tested for the further culture of Coker 312 cell suspension-derived protoplasts. Cell wall regeneration was observed within 3 days, but the first cell divisions were observed only after 2-3 weeks. This lagging phase was significantly reduced when a feeder layer technique was used. A highly friable callus was obtained after 6 weeks. Further regeneration is now in progress.

Since 1960, the FAO has promoted and supported network systems. In 1988 an Interregional Cooperative Research Network on Cotton was established with 15 participating countries; from Europe (Belgium, Bulgaria, France, Greece, Netherlands, Spain and Turkey), the Middle East (Iran, Israel, Pakistan, Syria) and North African Regions (Algeria, Egypt, Morocco, Sudan). The objective of this network is to promote voluntary exchanges of information, material and experimental data in selected subject matter fields, as well as effective cooperation in research on mutually selected topics. During the 2nd Consultation of this Network in Thessaloniki, Greece, the results of our research program were presented. At the same meeting a new working group, "Biotechnology in cotton," was created and Mrs. Peeters of the Laboratory of Tropical Crop Husbandry was selected as its coordinator. Since cotton biotechnology in the participating countries is in its infancy, a call is made for a concerted action between the members and assistance from advanced cotton biotechnologists elsewhere.

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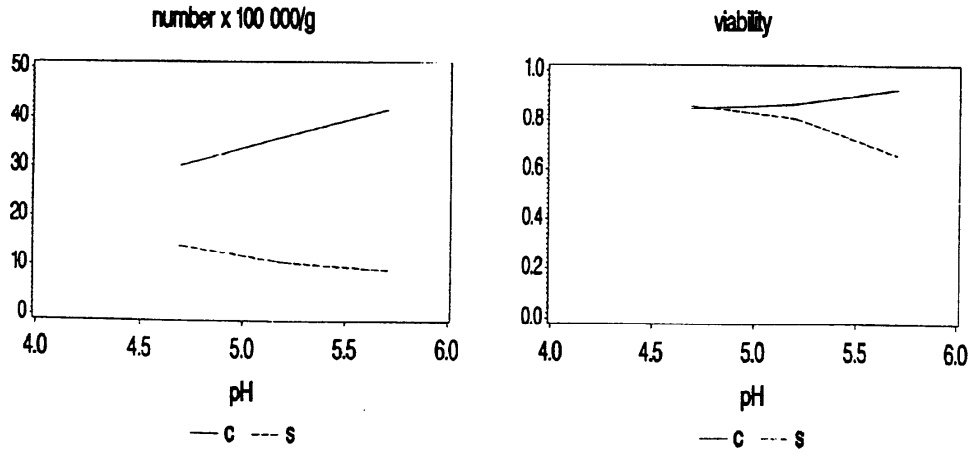
Figure 2: Influence of pH on protoplast yield and viability (ratio of number viable protoplasts to total number) of cotyledon (c)- and cell suspension (s)-derived Coker 312 protoplasts.



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