Characterization of somatic embryogenesis and plant regeneration in cotton (Gossypium hirsutum L.)

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Summary

Seventeen cultivars of cotton (Gossypium hirsutum L.) were evaluated for callus initiation and maintenance using 3 initiation media and 3 maintenance media. After a series of transfers of a 3% glucose media, calli were placed on a 3% sucrose medium. After several weeks calli were observed for the presence of embryo-like structures. Cultivars Coker 201 and Coker 315 were identified as embryogenic. Embryogenic callus has since been routinely obtained within 6 weeks by initiating callus on glucose media for 3-4 weeks followed by transfer to sucrose media. Histological examination has shown that embryos are derived from isodiametric, densely cytoplasmic cells and follow predictable patterns of development. Upon maturity, transfer to auxin-free media with reduced sucrose levels results in embryo germination. Regenerated plants can be transferred to greenhouse within 90 days of callus initiation.

Key words: Gossypium hirsutum - Somatic embryos - Regeneration - Morphogenesis - Genotype

Introduction

Plant regeneration is a critical step in the success of any crop improvement program entailing tissue culture techniques. Plant regeneration can be achieved in two ways: through organogenesis or through somatic embryogenesis. The latter is the preferred method, for two reasons. It is probable that plants derived from somatic embryogenesis are of single-cell origin (Haccius 1978). Thus, the plants will not be genetic chimeras, as is possible with those derived from organogenesis. Secondly, since nonzygotic embryos have no vascular connection with maternal tissue (Haccius 1978), in principle, they are more easily manipulated than plantlets derived through organogenesis.

Attempts to induce somatic embryogenesis revolve around two philosophies: manipulation of a wide range of inductive media and culture conditions in an attempt to incite an embryogenic response from a specific genotype, or evaluation of a large number of genotypes on a narrow range of 'inductive' media. The latter approach has been extended towards the identification of embryogenic genotypes within a species (Davidonis and Hamilton 1983). Preliminary results from our laboratory suggested that the Gossypium sp. germplasm collection contains much genotypic variation for callus initiation, proliferation, morphology and regeneration capacity. The objectives of this study were therefore to evaluate a wide range of genotypes for callus initiation and callus maintenance responses to several different media, to identify cotton genotypes that possess strong regeneration potential, and to characterize regeneration through histological analysis.

Materials and Methods

Callus Initiation and Maintenance: Seed were aseptically germinated on half-strength MS medium (Murashige and Skoog 1962) in darkness at 32 °C for 7 days. Germinated seeds were then transferred to a culture room at 27 °C with a 16/8 hour (2500 lx) day/night cycle. Hypocotyl longitudinal half-sections were obtained from 10-14 day-old seedlings and placed on 3% glucose media (Table 1). Eight to 10 hypocotyl sections, with 2 replicates, were placed on each of 3 initiation media. Medium 1 contained the salts of MS medium with 1 mg/L naphthalene acetic acid (NAA), 1 mg/L kinetin, 40 mg/L adenine, and 0.6% agar, pH 5.8 (Rani and Bhojwani 1976). Medium 2 contained MS salts with 2 mg/L indole acetic acid (IAA), 1 mg/L kinetin, and 0.6% agar, pH 5.7 (Smith et al 1977), and Medium 3 contained the salts of LS medium (Linsmaier and Skoog 1965) with 2 mg/L NAA, 1 mg/L kinetin and 0.85% agar (pH 5.7) (Davidonis and Hamilton 1983). Each medium contained glucose (3%) as the carbohydrate source. Explants were cultured on initiation media without subculture for 30 days, at 27 °C with 16/8 hour (2500 lx) day/night cycle. At the end of the callus initiation period, individual calli were weighed and transferred to one of three maintenance media. Medium 1 contained MS salts with 10 mg/L N6-(isopentenyl)-adenine (2IP) and 1 mg/L NAA (Price et al 1977), Medium 2M contained MS salts with 1 mg/L NAA, 0.5 mg/L kinetin, and 10 mM glutamine, and Medium 3M contained MS salts (lacking NH4NO3, but containing 3.8 g/L KNO3), with 1 mg/L NAA and 0.5 mg/L kinetin (Davidonis and Hamilton 1983).

After 30 days on maintenance media calli were visually scored for appearance, growth and general vigor and then transferred to media containing MS salts, 2 mg/L NAA, 1 mg/L kinetin, 3% glucose, and 0.3% Gel-rite (pH 5.8). Transfers were made at 7-10
Embryo Germination and Plant Regeneration: Mature cultivars (Table 1). Acala 1517 represented one extreme in which no callus growth was apparent on any of the media that were tested. For the remaining 16 cultivars, cell proliferation was clearly visible under the light microscope within 3-5 days. On average, Medium 11 produced approximately 30% more callus mass than either Medium 21 or 31, although this figure varied widely from genotype to genotype.

Table 1. Responses of different cotton cultivars to callus initiation. The callus mass (tWater weight) was determined after four weeks in culture.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>11</th>
<th>21</th>
<th>31</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acala 1517</td>
<td>no induction</td>
<td>no induction</td>
<td>no induction</td>
</tr>
<tr>
<td>Coker 201</td>
<td>47.50 ± 14.95</td>
<td>42.08 ± 18.23</td>
<td>55.25 ± 28.75</td>
</tr>
<tr>
<td>Coker 208</td>
<td>41.63 ± 22.71</td>
<td>40.90 ± 17.96</td>
<td>48.82 ± 9.58</td>
</tr>
<tr>
<td>Coker 310</td>
<td>26.49 ± 7.82</td>
<td>35.33 ± 19.79</td>
<td>29.64 ± 9.43</td>
</tr>
<tr>
<td>Coker 315</td>
<td>41.78 ± 9.50</td>
<td>49.00 ± 21.45</td>
<td>36.58 ± 9.16</td>
</tr>
<tr>
<td>Delacot 311</td>
<td>184.43 ± 79.32</td>
<td>216.55 ± 152.00</td>
<td>57.08 ± 27.61</td>
</tr>
<tr>
<td>Deltapine 61</td>
<td>50.29 ± 21.61</td>
<td>80.26 ± 26.28</td>
<td>56.96 ± 17.68</td>
</tr>
<tr>
<td>GSA 71</td>
<td>35.45 ± 18.00</td>
<td>67.19 ± 35.24</td>
<td>25.13 ± 12.27</td>
</tr>
<tr>
<td>Lankart 57</td>
<td>56.25 ± 28.40</td>
<td>82.69 ± 34.22 (R)</td>
<td>77.13 ± 26.93</td>
</tr>
<tr>
<td>McNair 235</td>
<td>15.52 ± 8.17</td>
<td>21.40 ± 12.27</td>
<td>22.40 ± 10.36</td>
</tr>
<tr>
<td>McPaw 145</td>
<td>98.53 ± 49.32</td>
<td>144.07 ± 21.40</td>
<td>138.44 ± 53.27</td>
</tr>
<tr>
<td>Quapaw</td>
<td>30.90 ± 21.63</td>
<td>51.90 ± 38.40 (S)</td>
<td>37.98 ± 24.74</td>
</tr>
<tr>
<td>RC10-3</td>
<td>19.08 ± 11.49</td>
<td>36.50 ± 23.15</td>
<td>36.42 ± 19.78</td>
</tr>
<tr>
<td>Stroman 254</td>
<td>45.21 ± 20.09</td>
<td>88.28 ± 24.90</td>
<td>54.82 ± 22.22</td>
</tr>
<tr>
<td>Tamcot CAMD-E</td>
<td>38.78 ± 16.00</td>
<td>60.65 ± 26.59</td>
<td>49.40 ± 23.83 (R)</td>
</tr>
</tbody>
</table>

R, root development; S, leaf or stem development

The explants were equal in size and their initial weights comprised 6.2 ± 2.0 mg (for this estimate N=29).

Histology: Embryos and embryogenic callus were fixed in formalin:acetic acid:ethanol:water (5:5:63:27) for 24 hours at room temperature. Fixed tissues were dehydrated in an ethanol/xylene series (Constabel 1982) and were embedded in paraffin (Paraplast TM 56-57°C). Ribbon sections of 8 um thickness were dried onto slides, dewaxed in xylene and rehydrated in a descending ethanol series. Sections were then triple-stained in safranin 0 and aniline blue-orange G (McDaniel et al 1982).

Embryo Germination and Plant Regeneration: Mature embryos were selected for germination on the basis of normal morphology. Germination was achieved by transferring to auxin-free MS media with 1 mg/L kinetin and 1.5% sucrose. This and subsequent manipulations were carried out at 27°C with a 16/8 hour (2500lx) day/night cycle. Once roots and leaves had developed the plantlets were transferred to Magenta boxes containing hormone-free 50% strength MS media with 1.5% sucrose (Fig 1C). After 1-2 weeks the plantlets were transferred into sterile vermiculte in a humid chamber, were gradually hardened-off, and were transferred to soil under normal greenhouse conditions (Fig 1D).

Results

Callus Initiation and Maintenance: A wide range of callus initiation and proliferation responses were observed among the hypocotyl explants of all 17 cultivars (Table 1). Acala 1517 represented one extreme in which no callus growth was apparent on any of the media that were tested. For the remaining 16 cultivars, cell proliferation was clearly visible under the light microscope within 3-5 days. On average, Medium 21 produced approximately 30% more callus mass than either Medium 11 or 31, although this figure varied widely from genotype to genotype.

The texture of the calli ranged from very hard and compact to watery and friable. Some degree of browning was observed in all cases. Regular and extensive root proliferation was observed in several cultivars (Table 1). We believe that the single isolated instance of shoot proliferation observed with cultivar Quapaw resulted from shoot emergence from meristematic regions of the explant rather than as a result of shoot organogenesis from callus.

Calli from each cultivar/ initiation media combination were subcultured onto each of the three maintenance media. The morphology of the callus grown on each of the maintenance media varied widely, with no obvious callus 'type' correlated to any specific media. Because callus types varied widely, even within a single plate, no attempt has been made to describe each callus type found on each combination of media. However, it was possible to rate the general growth and vigor of each group. Calli placed on Medium 3M generally showed the least vigorous growth. Medium 1M (2IP as the cytokinin) produced calli with an exceptionally vigorous and healthy appearance more often than either of the other maintenance media. Medium 2M produced consistently 'average' growth and vigor, with very little apparent effect from genotype.

Somatic Embryogenesis: The embryogenic response was restricted to cultivars Coker 201 and 315. Embryogenic callus was first observed as small sectors of a pale grey, compact, densely cytoplasmic callus emerging from a soft, yellowish to brown, friable callus. Emergence of embryogenic callus could not be correlated to any specific induction/maintenance media combinations. Repeated experiments showed that subculturing onto maintenance media was not necessary for the induction of embryogenic callus. Therefore, frequencies of embryogenicity were determined from callus initiated directly on MS medium with 3% glucose, 2 mg/L NAA and 1 mg/L kinetin and switched to 3% sucrose medium, with no intervening maintenance medium. Approximately 73% of Coker 201 and 64% of Coker 315 seed tested produced embryogenic callus (Table 2). When subcultured onto media containing MS salts, 2 mg/L NAA, 1 mg/L kinetin, and 3% sucrose, embryogenic callus continued to proliferate and produce somatic embryos. Figure 1A shows an example of typical embryogenic callus containing globular and heart-shaped embryos. These gradually develop into 'tulip-shaped' and mature embryos (several per plate)