INDUCTION OF MORPHOGENICALLY COMPETENT CALLUS AND SUSPENSION CELL CULTURES FROM LEAF EXPLANTS AND MATURE SEEDS OF DIGITARIA SANGUINALIS

R. A. GONZALES AND E. FRANKS

The Samuel Roberts Noble Foundation, Inc., Biomedical Division, Plant Cell Biology Section, P.O. Box 2180, Ardmore, Oklahoma 73402

(Received 20 December 1986; accepted 3 March 1987)

SUMMARY

Digitaria sanguinalis (crabgrass) has recently been introduced as a high quality forage crop. We report here a tissue culture system showing a high level of regeneration developed to aid in a breeding program. Two morphologically distinct types of callus, compact opaque and friable translucent, were induced from leaf blade explants and mature seeds when cultured on MS medium containing 0.9 μM 2,4-dichlorophenoxyacetic acid. Proline (25 mM) inhibited induction of callus but was required for continued maintenance. Plants were readily regenerated from the compact opaque callus. Selectively subcultured friable translucent callus continued to produce colony sectors of the morphogenically competent compact opaque callus when transferred to regeneration medium. Suspension cell cultures derived from callus or directly from leaf blade explants also produced regenerable callus.

Key words: regeneration; tissue culture; forage grass; gramineae.

INTRODUCTION

Efficient production of meat, milk, and wool depends on the availability of high quality plant material, whether provided as hay and silage or as forage. During the last decade, Digitaria sanguinalis (large or hairy crabgrass) has been developed as an alternative forage crop with excellent nutritional qualities (2,3). D. sanguinalis is found throughout the continental United States, although as a forage crop it is best adapted to the southern and southeastern portion of the country. Its useful range, however, can be extended because it responds well to irrigation. Several selections from natural stands have been made in the initial stages of a classical breeding program (3). It will now be possible to augment this program by merging the technologies of plant tissue culture and molecular biology.

Callus has been induced and plants regenerated in a number of other forage grasses [see (1) for a review]. Generally, regeneration frequency has been highest when the explant source was immature embryos. However, a number of reports have shown that regenerable callus was obtained from leaf explants (5-7,9,10,13) or from internode and peduncle explants (8). In every case, embryogenic callus was identified as compact opaque tissue. Upon transfer to regeneration media, these tissues formed embryos that matured and ultimately "germinated." Maintenance and proliferation of embryogenic cultures of most monocots and for some dicots, such as Glycine max, depend on the selective subculture of the compact opaque tissue, the friable translucent type callus being nonembryogenic (4,12). We report here a tissue culture protocol for the induction of morphogenically competent callus derived from leaf explants and mature seeds of D. sanguinalis.

As a consequence of an apparent interconvertability of a friable translucent-type callus with the morphogenically competent compact opaque-type callus, manipulation of calli and initiation of suspension cell cultures are greatly facilitated.

MATERIALS AND METHODS

Culture media. The basal salts medium used throughout this study was that of Murashige and Skoog (11), supplemented with 8.8 mM sucrose (MS). Bacto-agar (Difco, Detroit, MI) was added to 0.8% wt/vol where indicated.

Callus induction media consisted of MS agar supplemented with 0.9 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or 0.9 μM 2,4-D and 25 mM proline. Calli and suspension cultured cells were routinely maintained on MS plus 2,4-D and proline at 28°C.

Seed and seed germination. Seed (RR874) was provided by R. L. Dalrymple, SRNF, from Noble Foundation seed stocks. Seeds were surface sterilized by overnight exposure to chlorine gas (R. Stalhut, personal communication). A petri dish containing the seeds to be surface sterilized was placed in a glass desiccator along with a beaker containing 100 ml commercial bleach solution (5.25% sodium hypochlorite wt/vol). Three milliliters of concentrated HCl was slowly added to the bleach. The desiccator cover was immediately put in place and the unit set in a fume hood.

Surface-sterilized seeds were germinated aseptically under low light conditions (less than 500 lx) on MS agar.

Regeneration. Regeneration was initiated by transferring callus to MS agar and incubating under low light conditions...
THE EFFECT OF 2,4-D CONCENTRATION ON THE INDUCTION OF CALLUS FROM LEAF PLANTS OF *D. SANGUINALIS*

<table>
<thead>
<tr>
<th>μM 2,4-D</th>
<th>Number of Explants</th>
<th>Number with Callus</th>
<th>% Explants with Callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>290</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.45</td>
<td>290</td>
<td>12</td>
<td>4.1</td>
</tr>
<tr>
<td>0.90</td>
<td>290</td>
<td>104</td>
<td>35.9</td>
</tr>
<tr>
<td>1.80</td>
<td>290</td>
<td>57</td>
<td>19.7</td>
</tr>
<tr>
<td>3.60</td>
<td>290</td>
<td>127</td>
<td>43.8</td>
</tr>
</tbody>
</table>

*Leaf blade explants, 3- to 5-mm-long, were pretreated by culturing 1 wk on MS agar containing 0.9 μM 2,4-D. Explants were then transferred to MS agar containing the indicated level of 2,4-D and 25 mM proline and incubated at 28°C for 2 wk.*

RESULTS

Optimal induction and maintenance of callus from leaf blade explants of *D. sanguinalis* exhibited a series of specific requirements. Explants from mature leaves did not form callus in any of the culture treatments. Therefore, explants, distal to the ligule, were routinely taken from young (5 to 8-cm tall), aseptically grown plants.

Leaf blade explants were pretreated by culturing for 1 wk on MS agar supplemented with 0.9 μM 2,4-D. The explants were then transferred to fresh MS agar supplemented with 0.9 μM 2,4-D and 25 mM proline. Table 1 shows that this is the optimal 2,4-D concentration with ca. 36% of the explants forming callus after 3 wk. Although there was actually a higher frequency of callus production at 3.6 μM 2,4-D (Table 1), the callus formed was very watery, grew quite slowly, and was not considered of practical value.

If the pretreated explants were instead transferred to fresh MS agar containing the 2,4-D but without the proline, induction of callus occurred at a lower frequency (4% of the explants forming callus after 3 wk) and subsequent cell proliferation was greatly reduced or halted. Rapid cell proliferation was not reinitiated on subsequent transfer to fresh medium unless that medium contained 25 mM proline. Transfer of callus formed in the presence of proline to media lacking proline also resulted in reduced cell proliferation (data not shown). On the other hand, if the explants were not pretreated but placed directly onto the proline-containing medium, they quickly turned brown with no callus forming even after 4 wk in culture.

Whole mature seeds of *D. sanguinalis* could also be induced to form callus when cultured on MS agar supplemented with 0.9 μM 2,4-D. Morphogenically competent callus formed either directly from the seed without indication of germination or on stem and root segments from germinated seeds. As was observed for leaf blade explants, after callus induction, rapid cell proliferation stopped. Again, proliferation was not reinitiated unless transferred to fresh growth medium containing 25 mM proline. When whole seeds were cultured directly on MS agar supplemented with the 2,4-D and with 25 mM proline, callus formation was completely inhibited.

Callus formed from both the leaf blade explants and from mature seeds of *D. sanguinalis* which consisted of two morphologically distinct types (Fig. 1). One was friable and translucent, and the other was compact and opaque. The ability to form the compact opaque type callus was not lost even after extensive selective subculture of the friable translucent type callus.

Suspension cell cultures of *D. sanguinalis* comprised of single cells and small cell clumps were initiated by placing callus directly into liquid MS supplemented with 0.9 μM 2,4-D and 25 mM proline. Continued growth of the cultures resulted in the production of larger nodular opaque clumps (Fig. 2) similar in appearance to the compact opaque callus shown in Fig. 1. The nodular clumps were easily removed by sieving the culture through 100-μm mesh stainless steel screens. Subculture and subsequent growth of the filtered cells again resulted in the production of the nodular cell clumps after ca. 1 wk.

Similar suspension cell cultures were readily initiated from leaf blade explants. Explants were placed in liquid MS containing 0.9 μM 2,4-D and incubated for 1 wk with shaking. Proline was then added aseptically to 25 mM final concentration, and incubation continued.

Whole plants were routinely regenerated from both the compact opaque callus and the nodular opaque cell clumps derived from suspension cell cultures (Fig. 3). When the friable translucent type callus was transferred to regeneration medium, plants were obtained only after development of compact opaque callus sectors (Fig. 1). Addition of various concentrations of 6-benzylaminopurine or activated charcoal did not enhance regeneration frequencies (data not shown).

Regenerated plants were transferred to potting soil and placed in a greenhouse where they were allowed to set seed (Fig. 4). Seed was harvested and found to have normal viability (Table 2).

DISCUSSION

In an effort to develop the potential of biotechnology in a forage grass breeding program, a tissue culture protocol has been developed for *Digitaria sanguinalis* (large or hairy crabgrass). Morphogenically competent callus and suspension cell cultures are obtained when either leaf blade explants or mature seeds are cultured on media containing the growth

Table 2: Viability of Seeds Produced by *D. Sanguinalis* Plants Regenerated from Culture

<table>
<thead>
<tr>
<th>Plant</th>
<th>n</th>
<th>Viable</th>
<th>% Viable</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>115</td>
<td>112</td>
<td>97.4</td>
</tr>
<tr>
<td>2</td>
<td>101</td>
<td>98</td>
<td>97.0</td>
</tr>
<tr>
<td>3</td>
<td>82</td>
<td>64</td>
<td>78.0</td>
</tr>
<tr>
<td>4</td>
<td>146</td>
<td>124</td>
<td>84.9</td>
</tr>
<tr>
<td>Control</td>
<td>210</td>
<td>164</td>
<td>78.1</td>
</tr>
</tbody>
</table>

*Number of seeds tested for viability.

Viability was scored as the ability to germinate in a seed germination test.

Control seeds were from the original seed stock.