ESTABLISHMENT OF FRIABLE EMBRYOGENIC (TYPE II) CALLUS FROM IMMATURE TASSELS OF ZEA MAYS (POACEAE)\textsuperscript{1}

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Type II callus cultures were initiated from immature tassels of a maize genotype with an A188/B73 genetic background using N6 medium containing 1.0 mg/liter 2,4-D, 100 mg/liter casamino acids, 25 mm proline, and 0.2% phytagel\textsuperscript{TM}. Inclusion of 10 \(\mu\)M AgNO\textsubscript{3} in this medium significantly increased the frequency and vigor of the type II callus response. Friable calli emerged from these explants after two consecutive 2-week subculture intervals. Tassels from 10 to 30 mm long were capable of producing type II cultures. The plants regenerated from these cultures were green and indistinguishable from plants regenerated from immature embryo-derived calli.

Embryogenic maize calli are commonly grouped into two distinct classifications based on their morphology. Type I calli are characterized as compact cultures where the somatic embryos are often considered morphologically complex and organized (Armstrong and Green, 1985). Type II calli are characterized as friable where the somatic embryos are discretely arranged throughout the culture (Armstrong and Green, 1985). This friable nature makes type II calli the preferred inoculum for the initiation of embryogenic suspension cultures, which are currently a desirable target tissue in the transformation of maize through microprojectile bombardment (Fromm et al., 1990; Gordon-Kamm et al., 1990).

There have been several recent reports describing the effect of AgNO\textsubscript{3}, an ethylene antagonist (Beyer, 1976), on induction of type II embryogenic calli from immature embryo explants of maize inbreds A188 (Vain, Flament, and Soudain, 1989; Vain, Yean, and Flament, 1989) and B73 and its derivatives (Songstad, Armstrong, and Petersen, 1991). Various ethylene antagonists also promoted plant regeneration from type I callus cultures of maize inbreds Pa91 and H99 (Songstad, Duncan, and Widholm, 1988). AgNO\textsubscript{3} appears to promote callus induction by preventing endogenous ethylene (generated by explant wounding and in response to in vitro culture) from affecting the embryogenic response.

A substantial amount of wounding is usually involved in the initiation of callus cultures from unemerged immature tassel tissues. From our previous experience, cutting a 25- to 30-mm tassel (composed of one central spike and several side branches) into 1.0- to 1.5-mm segments resulted in over 100 separate tissue pieces for culture initiation. A recent report using this size of immature tassel explant resulted in only compact type I embryogenic callus cultures using a Murashige and Skoog (MS) - based medium containing 1 to 2 mg/liter 2,4-D, 25 mm proline, 195 \(\mu\)M AgNO\textsubscript{3}, and 0.8% agar (Pareddy and Petolino, 1990). However, Rhodes, Green, and Phillips (1986) obtained a single friable type II embryogenic callus from their tassel culture efforts. We now report the use of an N6-base callus induction medium containing 1 mg/liter 2,4-D, 25 mm proline, 10 \(\mu\)M AgNO\textsubscript{3}, and 0.2% phytagel\textsuperscript{TM} for the initiation of friable type II cultures from immature tassels.

MATERIALS AND METHODS

Greenhouse and field-grown Zea mays L. plants from A188, B73, B73 \(\times\) A188, and a “High Type-II” (Hi II) genotype (derived from progeny of an A188 \(\times\) B73 cross) were used as the source of tassels. Approximately 4–6 weeks after planting, plants were collected and outer leaves removed. The inner leaves were aseptically removed, and unless otherwise mentioned, tassels with a length of 10 to 30 mm were retained (Fig. 1). The tassels were then pooled and homogenized by cutting into 1.0- to 1.5-mm segments and cultured (175 tassel sections per plate) on a modified MS (Murashige and Skoog, 1962; modified by Green and Phillips [1975] by the addition of a 1 mm asparagine) or N6 (Chu et al., 1975) medium containing 2% sucrose, 0.5 mg/liter thiamine, 1.0 mg/liter 2,4-D, 0 or 100 mg/liter casamino acids, and 0 or 25 mm proline (Armstrong and Green, 1985). In a second experiment, up to 100 \(\mu\)M AgNO\textsubscript{3} was added to the above stated N6 medium containing 1 mg/liter 2,4-D, 100 mg/liter casamino acids, and 25 mm proline (abbreviated as N6 1-100-25) to assess its affect on type II callus induction from Hi II tassels. The media were solidified with 0.2% phytagel\textsuperscript{TM} or 0.7% Difco-Bacto\textsuperscript{TM} agar, and cultures were incubated in the dark at 28 C. After 2 weeks of incubation, the cultures were transferred to the same respective medium so that 20 tassel segments were on each plate. Cultures were visually assessed for type II calli after another 3 weeks of incubation. Plants were regenerated according to the protocol described by Armstrong and Green (1985).

RESULTS

Initial experiments involved comparison of the in vitro culture response of Hi II immature tassel explants cultured on N6 and MS media with or without 100 mg/liter casamino acids and 25 mm proline (all media combinations

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TABLE 1. Effect of N6 vs. MS basal medium and presence or absence of proline on induction of type II cultures from maize tassel segments

<table>
<thead>
<tr>
<th>Media combination</th>
<th>Type II response (%)</th>
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<tbody>
<tr>
<td>MS + 0 proline</td>
<td>0.7 a</td>
</tr>
<tr>
<td>N6 + 0 proline</td>
<td>0.4 a</td>
</tr>
<tr>
<td>MS + 25 mm proline</td>
<td>4.0 ab</td>
</tr>
<tr>
<td>N6 + 25 mm proline</td>
<td>5.5 b</td>
</tr>
</tbody>
</table>

* MS and N6 media solidified with 0.7% agar.
  
  b Average percentage of tassel segments forming type II calli from 12 replications. Approximately 8,400 tassel pieces (from 23 immature tassels) were cultured at a density of approximately 175/plate. Numbers associated with the same letter are not significantly different according to Duncan's New Multiple Range Test (5% level).

Immature tassels of various sizes (ranging from 10 to 40 mm long) were cultured on agar solidified (0.7%) N6 1-100-25 to determine if the developmental stage influenced the type II culture response. There was no significant difference among the type II callus induction rates for those explants from 10- to 30-mm long tassels (approximately 4.0% to 5.5% response). However, this response rate decreased to nearly 1.0% as the tassels approached a length of 40 mm. Therefore, in all future experiments, those tassels 10 to 30 mm long were pooled, randomly dissected into 1.5-mm-long segments, and used as a common explant source for culture induction.

Microscopic examination showed that the friable calli originating from these cultures arose from tumid regions of the tassel segments. The onset of this response was the development of clusters of young somatic embryos arising from discrete meristematic regions among the explants during the first 2-week culture period (Fig. 2). This initial embryogenic response was followed by the establishment of type II callus cultures (Fig. 3), which were indistinguishable from those initiated from immature embryos. The plants regenerated from both callus sources were identical.

AgNO₃ was then added to N6 1-100-25 and used to assess if control of ethylene action would promote the type II culture induction frequency from immature tassel explants of this genotype. The 10 µM AgNO₃ treatment promoted a significant increase in the number of explants producing type II callus when compared to the control (Table 2). While the 50- and 100-µM treatments also gave higher type II induction frequencies than the control, the differences were not statistically significant. This indicates
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Table 2. Effect of AgNO₃ on type II callus response from Hi II tassel segments after two consecutive 2-week culture intervals on N6 1-100-25

<table>
<thead>
<tr>
<th>AgNO₃ (μM)</th>
<th>Type II response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>19 a</td>
</tr>
<tr>
<td>10</td>
<td>59 b</td>
</tr>
<tr>
<td>50</td>
<td>33 a</td>
</tr>
<tr>
<td>100</td>
<td>37 a</td>
</tr>
</tbody>
</table>

Silver nitrate filter sterilized into N6 1-100-25 containing 0.2% phytagel after autoclaving.

Percent of tassel segments that produced type II callus cultures. Numbers represent average values from approximately five replications. Approximately 3,500 tassel pieces (from 12 immature tassels) were cultured at a density of nearly 175 pieces/plate. Means with identical letters are not significantly different according to Duncan’s New Multiple Range Test (5% level).

that AgNO₃ concentrations higher than 10 μM may not be optimal for inducing type II calli under the described culture conditions.

This N6 1-100-25 medium formulation containing AgNO₃ did not promote the type II response from tassel explants of A188, B73, or the B73 × A188 F₁ hybrid (approximately 25, 20, and 15 tassels from A188 and B73 × A188, respectively, were tested in two separate experiments). One type II culture was obtained following the incubation of nearly 5,000 A188 tassel pieces (from 23 tassels) on medium without AgNO₃. Furthermore, the type I callus induction rate was not substantially improved by the presence of AgNO₃. For example, approximately 2%, 4%, and 5% of the B73, A188, and B73 × A188 tassel explants, respectively, produced type I callus in the absence of AgNO₃. The response from these genotypes ranged from approximately 1% to 4% in the presence of 10 or 100 μM AgNO₃.

**DISCUSSION**

The onset of the friable type II response from Hi II immature tassel tissues was preceded by the development of discrete early-stage somatic embryos from the explant during the first 2-week culture period. This is similar to the development of inflorescence-derived embryogenic callus tissue from Schizachyrium scoparium where somatic embryos arose from localized meristematic masses (Songstad, Chen, and Boe, 1986). It appears that during the second 2-week culture period, these early embryogenic structures develop into recognizable type II callus. The second incubation period is necessary to allow the responding cells the extra time required for the callus to form, especially in the absence of AgNO₃.

Our data suggest that genotype is a limiting factor regarding induction of type II cultures from maize immature tassel tissues. Only the Hi II genotype demonstrated an efficient type II callus response from tassel explants. There was also a type II response from A188, but this was isolated to a single callus event, and additional attempts to isolate friable cultures from tassel explants of this genotype were not successful.

The Hi II genotype is an A188 × B73 derivative originally selected for enhanced production of type II callus from immature embryo explants on N6 1-100-25 medium. In this report similar results were obtained using immature tassels that were 10 to 30 mm long (approximately 5 weeks after planting). However, tassels larger than this showed a decreased ability to produce type II calli (data not shown). This decline in the embryogenic culture initiation rate is likely due to physiological changes within the explants during the course of continued development. Our results agree with previous reports that a proper explant stage is required for optimal induction of callus from immature embryo tissues (Green and Phillips, 1975; Armstrong and Green, 1985).

In our initial experiments, a 5.5% type II callus induction response was obtained from tassel segments cultured on N6 1-100-25 medium (no silver nitrate) solidified with agar (Table 1). However, a 19% type II response was obtained from tassels cultured on phytagel solidified form of this medium (Table 2). This agrees with our previous results with immature embryos of B73, and its derivatives (Songstad, Armstrong, and Petersen, 1991), where the type II callus induction rate was enhanced by culturing on N6 1-100-25 medium solidified with phytagel (as compared to the agar solidified control).

Using the Hi II genotype, no significant differences between MS and N6 basal salts were detected. This contradicts the conclusions by Rhodes, Green, and Phillips (1986) that MS medium induced more positive culture responses than the N6 formulation described by Armstrong and Green (1985). Perhaps this discrepancy is due to the use of the Hi II genotype in the present study, which was specifically selected for enhanced response on N6 1-100-25.

The role that proline has in promoting the type II response from immature tassel cultures is not currently known. However, this need for proline agrees with other published reports where it promoted the induction of embryogenic (type I) cultures from Oh43 immature tassel cultures (Pareddy and Petolino, 1990) and was vital for the establishment of type II cultures from A188 immature embryo explants (Armstrong and Green, 1985).

Corn is one of several species where control of ethylene results in a superior in vitro culture response. However, ethylene is required or its control has no effect on the culture response from Dactylis glomerata (Songstad et al., 1989), Hordeum vulgare (Cho and Kasha, 1989), Lilium speciosum (van Aartrijk, Blom-Barnhoorn, and Bruinsma, 1985), and Solanum carolinense (Reynolds, 1987). These examples indicate that the effect of ethylene on in vitro cultures of other species has to be assessed on an individual basis.

Aside from immature embryo and tassel tissues, leaf base (Chang, 1983; Conger et al., 1987; Ray and Ghosh, 1990), glume (Suprasanna, Rao, and Reddy, 1986), immature ears (Pareddy and Petolino, 1990), and mature embryo (Wang, 1987) explants have been used to initiate callus cultures of maize. However, these cultures were described as compact (likely a type I culture), limited in ability to induce type II calli or organogenic. Culturing these explants on medium with an optimal level of AgNO₃ may result in enhanced production of friable cultures, although this may depend upon using the proper genotype as well as the developmental stage of the explant.
LITERATURE CITED


