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$AgNO_3$ increases type II callus production from immature embryos of maize inbred B73 and its derivatives

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ABSTRACT

Incorporating 10 to 100 μM $AgNO_3$ into Phytagel™ (0.2%) solidified N6 medium containing 1 mg/L 2,4-D, 100 mg/L casamino acids and 25 mM proline (N6 1-100-25) promoted type II callus production from cultured *Zea mays* L. immature embryos of FRB73, B73 X A188 and a proprietary B73 BC6 genotype. Under these conditions, approximately 15, 80 and 80% of the respective FRB73, B73 X A188 and B73 BC6 explants produced type II calli after 2 to 3 weeks incubation in the dark at 28 C. In the absence of $AgNO_3$, the type II culture response from B73BC6 immature embryos was 25% on N6 1-100-25 solidified with Phytagel™ (0.2%) as compared to 0% for that solidified with 0.8% agar. Duncan's medium was tested using 10 to 100 μM $AgNO_3$ and generally promoted type I callus initiation, although up to 6% of the explants produced type II cultures in the presence of 0.2% Phytagel™. Ethylene emanation rates of up to 370 and 115 nL g-1 h-1 were detected from B73 X A188 immature embryos and calli, respectively, cultured on N6 1-100-25.

INTRODUCTION

It is becoming increasingly apparent that control of ethylene is important in maize tissue culture. One way to control the effect of ethylene is by using $AgNO_3$ (Beyer, 1976) which competes with ethylene for its binding site, but does not interrupt ethylene biosynthesis. Songstad et al. (1988) used $AgNO_3$ and another ethylene action blocker, norbornadiene, to promote plant regeneration from H99 and Pa91 type I callus cultures. Vain et al. (1989a, 1989b) demonstrated that $AgNO_3$ enhances initiation of type II cultures from A188 immature embryos. However, the role of ethylene on callus induction from immature embryos of other genotypes has yet to be reported. Therefore, we describe the effect of $AgNO_3$ on induction of type II cultures from the agronomically elite maize inbred line B73, the F_1 hybrid B73 X A188 and a proprietary B73 X A188 backcross line. The interaction of various media combinations and solidification agents were also examined.

MATERIALS AND METHODS

Field or greenhouse grown *Zea mays* L. plants (B73, FRB73, and B73 BC6) were used as sources of immature embryos (1.0 to 2.0 mm long). FRB73 was obtained from Illinois Foundation Seeds (Champaign, IL) and B73 and A188 obtained from the University of Minnesota (C.M. Donovan, Dept. of Agronomy and Plant Genetics). B73 BC6 is a proprietary highly regenerable version of B73 developed by backcrossing a regenerant from an A188 X B73 F_2 culture for 6 generations with B73 and selecting at each generation for high frequency type II culture initiation (Armstrong, unpublished). Cultures were established by incubating explants on N_6 medium containing 1.0 mg/L 2,4-D, 100 mg L^{-1} casamino acids and 25 mM proline (hereafter referred to as N_6 1-100-25; Armstrong and Green, 1985) or on D medium, a modified N_6/B_5 medium as described by Duncan et al. (1985). For both media, all possible combinations of 0, 10, 50, or 100 μM $AgNO_3$ with 0.2% Phytagel™ (Sigma) or 0.8% TC agar (Sigma) were created and arranged in a randomized complete block design. A 2.0 mg/ml aqueous $AgNO_3$ stock solution was prepared and appropriate amounts filter sterilized into the media after autoclaving. These plates were stored in the dark immediately after the media solidified and briefly exposed to light during the explanting process. All cultures were grown (in the dark) with embryonic axis contacting the medium for 21 d at 28 C. At this time the cultures were scored for type I vs type II callus production. Callus was then subcultured onto N_6 1-100-25 ± 50 μM $AgNO_3$ containing 0.2% Phytagel™ for stock culture establishment and eventually transferred through a regeneration protocol as described by Armstrong and Green (1985).

Ethylene emanation was assessed from 3 to 5 immature embryos and established callus cultures grown in 25 ml erlenmeyer flasks containing 12 ml N6 1-100-25 medium solidified with 0.2% Phytagel™. Sterile septa were placed on the flasks for 1 to 2 hours to capture the ethylene, and 1.0 ml gaseous samples collected from the headspace and injected into a Varian Model 3400 gas chromatograph equipped with a Poropak R column and flame ionization detector. The septa were removed immediately after ethylene determination and replaced with aluminum foil to permit normal gas exchange. This procedure was repeated each day ethylene was assessed and the immature embryos were not disturbed during the quantification process. At the end of the timecourse (day 15), the final fresh weights of the cultures were obtained and intermediate weights estimated based on linear growth (Figure 1; Songstad et al., 1990). All samples were quantified as nL ethylene g^{-1} fresh weight h^{-1}.

RESULTS and DISCUSSION

Ethylene release from B73 X A188 immature embryos

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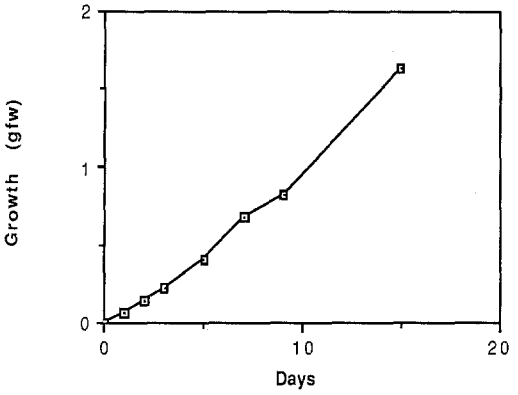

Fig. 1. Growth of B73 X A188 calli following 15-d culture on N6 1-100-25 (0.2% gelrite). Each point represents the average g fresh weight increase from 4 replications.

immediately after isolation was too low to measure (less than 0.2 nL g^{-1} h^{-1}). However, it increased sharply and reached a peak of 370 nL g^{-1} h^{-1} after 10 d culture (Table 1). The emanation rate then decreased to approximately 10 nL g^{-1} h^{-1} after 15 d incubation.

Similar results occurred when ethylene emanation was assessed from 1-year old B73 X A188 callus cultures. The initial emanation rate was approximately 2.2 nL g^{-1} h^{-1} and increased to 115 nL g^{-1} h^{-1} 4-d following subculturing (Table 2). The final (day 15) ethylene release rate was approximately 10 nL g^{-1} h^{-1}. Our maximum ethylene release after 4 d culture is nearly 15-fold greater than that reported by Songstad et al. (1988) from maize inbred Pa91 type I calli (maximum of 7.4 nL g^{-1} h^{-1} after 3 d culture). This is likely due to either differences in genotype, media composition, or type I vs type II tissue.

Table 1. Ethylene emanation from B73 X A188 immature embryos cultured on N6 1-100-25 for 15 days.

Days in Culture	Ethylene (nL g^{-1} h^{-1}) †	
0	>0.2	b
6	151	a
10	370	a
12	79	b
15	13	b

† Average from 4 replications. Different letters represent significant differences according to Duncan's New Multiple Range Test (5% level).

A significantly greater number of type II embryogenic calli were obtained from B73 X A188 immature embryos cultured on N6 1-100-25 containing 0.2% Phytagel™ and 10 to 100 µM $AgNO_3$ vs the control (Table 3). Across all the $AgNO_3$ treatments, approximately 80% of the immature embryos produced friable type II calli. These results agree with the findings of Vain et al. (1989 a,b) regarding type II callus induction from A188 immature embryos.

Table 2. Ethylene emanation from B73 X A188 calli cultured on N6 1-100-25 for 15 days.

Days in Culture	Ethylene (nL g^{-1} h^{-1}) †		
0	2	a	
0.3	44	a	
1	32	a	
2	41	a	
3	99	c	b
4	115		c
7	52	a	b
12	8	a	
15	12	a	

† Average from 4 replications. Means with different letters are significantly different according to Duncan's New Multiple Range Test (5% level).

An increase in the induction frequency of type II calli was obtained when B73 BC6 immature embryos were cultured on N6 1-100-25 containing 0.2% Phytagel™ and 10 to 100 µM $AgNO_3$ (Table 4). In fact, all of the explants grown on medium containing 100 µM $AgNO_3$ produced type II calli, compared to 25% in the absence of $AgNO_3$. Culturing these immature embryos on agar solidified N6 1-100-25 without $AgNO_3$ did not produce any type II calli; however, adding up to 100 µM $AgNO_3$ restored the embryogenic capacity to approximately 80% (Table 4)

Type II calli were also initiated from FRB73 immature embryos cultured on N_6 1-100-25 containing 10 to 50 µM $AgNO_3$ (Figure 2) and solidified with 0.2% Phytagel™ (Table 4). Approximately 10 to 20% of the explants produced friable calli under these conditions, compared to a complete lack of embryogenic response from the no $AgNO_3$ control. Type II cultures were also initiated on agar-solidified N6 1-100-25 containing $AgN0_3$, but at a lower frequency (6%). The

Table 3. Effect of $AgNO_3$ addition to N6 1-100-25 on callus induction from B73 X A188 immature embryos.

$AgNO_3$ (µM)	% Type II Cultures †	
0	9.1	a
10	81.3	b
50	77.6	b
100	82.5	b

† Average from 7 replications of 20 immature embryos per plate. Means with different letters are significantly different according to Duncan's New Multiple Range Test (5% level). N6 1-100-25 solidified with 0.2% Phytagel™.

Table 4. Effect of $AgNO_3$ and solidification agent on type II callus induction from B73 BC6 and FRB73 immature embryos cultured on N6 1-100-25.

	B73 BC6		FRB73	
$AgNO_3$ (µM)	Agar†	Phytagel™†	Agar	Phytagel
0	0 b	25.0 c*	0 a	0 b
10	75.0 a	61.7 b	4.1 a	20.4 a
50	83.3 a	76.7 b	6.1 a	14.3 a
100	81.7 a	100 a	6.1 a	4.1 b

† Agar (0.8%) Phytagel™ (0.2%)

* Data represents average percentage of explants forming type II cultures (80 immature embryos plated per treatment as 4 reps of 20). Means with different letters are significantly different according to Duncan's New Multiple Range Test (5% level).

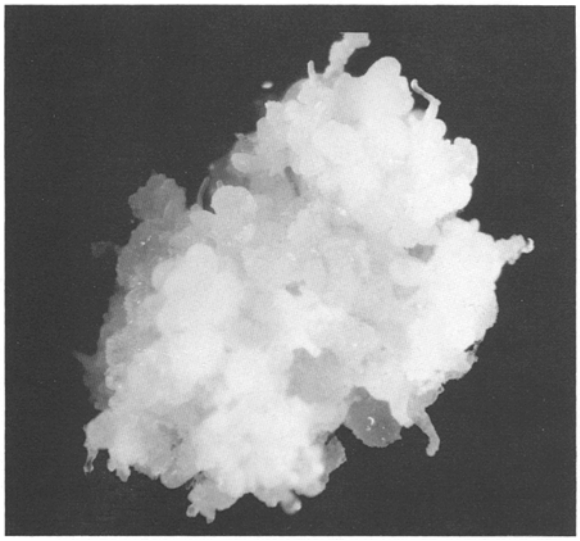

Fig. 2. FRB73 type II callus following 5 subculture cycles on N6 1-100-25 (0.2% phytagel) containing 50 µM $AgNO_3$ (15 X).

experiment was repeated and nearly identical results were observed (nearly 10% of the FRB73 immature embryos produced type II cultures). In comparison to FRB73, a 3% type II response was observed using B73 under these conditions. The reason for this decrease is not currently known.

The beneficial effect of $AgNO_3$ was then tested in conjunction with D medium. $AgNO_3$ promoted a slight increase in the type II callus induction frequency in B73 BC6 and FRB73 explants (3 and 6%, respectively; Table 5). However, this is a 95% (for B73 BC6) and 70% (for FRB73) lower type II induction frequency than that observed with the N6 1-100-25 formulation containing $AgNO_3$. The type II response from this medium did not appear to be linked with agar or Phytagel™. On the other hand, Duncan's medium containing up to 100 µM $AgNO_3$ promoted the type I response as compared to the N6 1-100-25 counterpart (data not shown).

Table 5. Effect of $AgNO_3$ and solidification agent on type II callus induction from B73 BC6 and FRB73 immature embryos cultured on D medium.

	B73 BC6		FRB73	
$AgNO_3$ (µM)	Agar†	Phytagel™†	Agar	Phytagel™
0	0 a	0 a*	0 a	0 a
10	1.7 a	0 a	0 a	6.1 a
50	1.7 a	0 a	2.0 a	2.0 a
100	3.3 a	1.7 a	0 a	4.1 a

† Agar (0.8%) Phytagel™ (0.2%)

* Data represent average percentage values of embryos producing type II cultures (80 immature embryos plated per treatment as 4 reps of 20). Means with same letter are not significantly different according to Duncan's New Multiple Range Test (5% level).

The optimal concentration of $AgNO_3$ for promoting type II callus cultures of B73 and its derivatives has not yet been identified. A positive response was observed within a range of 10 to 100 µM (1.8 to 18.0 mg/L), which agrees with what Vain et al. (1989b) reported with regards to type II cultures from A188 (5 to 10 mg/L). Determining the minimal amount of Ag+ necessary for inducing this culture response may be essential if it is required to maintain subcultured calli with a type II morphology. Early results indicate that the FRB73 type II calli require $AgNO_3$ in the medium or else they develop type I characteristics. It is still unknown whether long-term exposure to $AgNO_3$ will result in problems with heavy metal toxicity.

Ordinarily one expects less than 1% of B73 immature embryos to initiate type II calli (Tomes and Smith, 1985). From our experiences, we expect approximately 0.1% of the explants to produce this type of callus when cultured on Phytagel™ solidified N6 1-100-25. The addition of $AgNO_3$ to N6 1-100-25 solidified with 0.2% Phytagel™ enables 10 to 20% of the FRB73 embryos to produce such friable cultures. In our lab, we are now routinely using this $AgNO_3$ medium to initiate type II cultures. This is certainly a breakthrough regarding tissue culture manipulation of B73 and its derivatives.

The role of ethylene in corn tissue culture is becoming increasingly apparent. However, this is not necessarily the case with cultures from other species. For instance, various ethylene antagonists were used to promote shoot regeneration in *Brassica campestris* ssp. chinese (Chi and Pua, 1989), *Triticum aestivum* and *Nicotiana plumbaginifolia* (Purnhauser et al., 1987) and to increase somatic embryo formation in *Daucus carota* (Roustan et al., 1990). However, ethylene either had no detrimental effect or promoted regeneration and/or embryogenesis in *Dactylis glomerata* (Songstad et al., 1989), *Lilium speciosum* (van Aartrijk et al., 1985), *Solanum carolense* (Reynolds, 1987) and *Medicago sativa* (Meijer and Brown, 1988). This indicates that the response to ethylene antagonism needs to be addressed with each species. Furthermore, genotype differences within species may exist and should be considered.

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