

# Regulation of Tracheary Element Differentiation by Exogenous Methionine in Callus of Soybean Cultivars

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## ABSTRACT

The relationship between the induction of tracheary element differentiation and exogenous methionine was examined in agar cultures of soybean callus initiated from Wayne and Clark 63 cv., respectively, of *Glycine max* L. Although the Wayne is a normal cv. of soybean, seedlings of the Clark are known to exhibit abnormal growth at 25°C due to excessive ethylene biosynthesis at this temp. (Samimy and LaMotte, 1976). Wayne callus showed increased xylogenesis in the presence of exogenous methionine (0.025  $\mu$ M) in comparison to IAA-KN controls at both 20°C and 25°C. Clark 63 callus produced greater numbers of tracheary elements in response to exogenous methionine only at 25°C. The induction of xylem differentiation was independent of the maintenance temp. of the stock cultures of both cv. Xylogenesis initiated by an IAA-KN medium was inhibited by the addition of AgNO<sub>3</sub> (20 mg l<sup>-1</sup>) to the extent of 76.5% in the Wayne in the Clark 63. The inhibitory effect was partially reversed by the addition of methionine (0.025  $\mu$ M) to the IAA-KN-AgNO<sub>3</sub> medium. These data support the hypothesis that xylogenesis *in vitro* involves auxin, cytokinin, and ethylene.

Key words: differentiation, xylogenesis, methionine, ethylene, *Glycine max* L. cv. Clark 63, *Glycine max* L. cv. Wayne, and soybean callus.

## INTRODUCTION

Since the addition of methionine to a xylogenic culture medium strongly stimulated tracheary element differentiation in explants of lettuce pith, the hypothesis was advanced that the enhanced biosynthesis of ethylene arising from the methionine substrate plays a role in xylogenesis (Roberts and Baba,

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1978). Experiments by Samimy and LaMotte (1976, 1978) with the Clark cv. of soybean indicated that this plant material might yield callus suitable for testing the hypothesis regarding ethylene and xylogenesis. The lack of normal hypocotyl elongation of Clark seedlings grown at 25°C was associated with excessive ethylene biosynthesis at this temp. Clark seedlings produced normal growth at 20°C and 30°C, and ethylene biosynthesis was considerably lower at these temp. in comparison to 25°C (Samimy, 1978a, b; Samimy and LaMotte, 1976, 1978). To our knowledge no prior investigations have employed callus derived from the Clark cv. Our present data give indirect evidence that the callus cells of the Clark 63 cv. express this trait, i.e., a 25°C temp.-dependent ethylene biosynthesis.

Studies with agar cultures of soybean callus have shown the suitability of this plant material for the *in vitro* induction of tracheary element differentiation (Torrey, 1968; Fosket and Torrey, 1969). In particular, an NAA-KN medium was developed by Fosket and Torrey (1969) for the proliferation of cells in the absence of tracheary element differentiation. A "tracheid-free medium" is useful for the production of callus for xylogenic expt..

The present study involves the induction of xylogenesis in explants of soybean callus obtained from the Wayne and Clark 63, respectively, on a methionine-enriched medium. Expt. were conducted at 20°C and 25°C, respectively. The Wayne, a normal cv. of soybean, provided a control for results obtained with the Clark 63 callus. The incorporation of AgNO<sub>3</sub> into the medium provided further evidence on the postulated role of ethylene in xylogenesis.

## MATERIALS AND METHODS

Soybean seeds were surface sterilized by immersion in ethanol (70% v/v) for 2 min followed by 10 min in a 10% (v/v) aqueous soln of Clorox containing 5.25% (w/v) sodium hypochlorite as the active agent. The seeds were passed through 4 rinses of sterile double-distilled H<sub>2</sub>O, and the seeds were retained in the final rinse for approx. 12 h prior to explant prep. A single explant was removed from each cotyledon by boring the seed with a 5 mm I.D. cork borer. The explants were rinsed twice with sterile double-distilled

H<sub>2</sub>O, blotted on sterile Whatman No. 1 filter paper, and cultured individually in scintillation vials (15 ml capacity) containing 10 ml of medium. The vials, capped with aluminum foil, were placed in glass culture jars (10 vials per jar). The jars, enclosed with foil, were incubated at the specified temp..

Freshly prepared explants were initially placed on a B5 medium (Gamborg, et al., 1976) supplemented with 2,4-D (1 mg l<sup>-1</sup>), sucrose (3% w/v), and Bacto-Agar (1% w/v). This medium was designated by 1B5. After 10 d culture the resulting callus was excised from the explant and subcultured on a B5TF medium consisting of a basal B5 mixture supplemented with  $\alpha$ -NAA (1.86 mg l<sup>-1</sup>), KN (0.0022 mg l<sup>-1</sup>), sucrose (3% w/v), and Bacto-Agar (1% w/v). After 7 d culture on the B5TF medium, the resulting callus was again subcultured on a B5TF medium. on 24 d from the time of explant prep., the actively-growing callus was subcultured on B5D. The latter medium consisted of a basal B5 mixture supplemented with IAA (5 mg l<sup>-1</sup>), KN (0.1 mg l<sup>-1</sup>), sucrose (3% w/v), and Bacto-Agar (1% (w/v)). When specified, the B5D medium was supplement with L-methionine (3.7 ug l<sup>-1</sup>) and AgNO<sub>3</sub> (20 mg l<sup>-1</sup>). The callus remained on the experimental B5D medium for 7 d. In resumé, the following steps were used sequentially: callus initiation on 1B5 (10 d), tracheid-free conditions on B5TF (7 d; 7 d), and xylogenic test conditions on B5D (7 d). Each expt therefore lasted a total of 31 d. In expt involving the addition of AgNO<sub>3</sub>, Ca(NO<sub>3</sub>)<sub>2</sub> was substituted for CaCl<sub>2</sub> in the basal medium in order to avoid the formation of a AgCl ppt. All media were autoclave sterilized under standard conditions Methionine and AgNO<sub>3</sub> were sterilized by Millipore filtration at room temp. and added to the warm autoclaved media.

At the termination of each expt the callus samples were weighed and macerated in preparation for tracheary element cell counts. The counting procedure, devised by Brown and Rickless (1949), was adapted for xylem cell analysis by Dalessandro and Roberts (1971). Samples were placed individually in vials containing 1 ml of a maceration fluid composed on equal parts of chromic acid (5% w/v) and HCl (5% v/v) for 24 h at room temp. The maceration fluid was removed and replaced with 1 ml dist. H<sub>2</sub>O. The disrupted sample was drawn repeatedly into a hypodermic syringe (2 ml) fitted with a 22G needle. The cell suspension was brought to a total vol.

of 2 ml, and 1 ml of the thoroughly-mixed sample was transferred to a Sedgewick-Rafter plankton counting chamber. The no. of tracheary elements were counted in 5 optical fields with the aid of a Whipple eyepiece micrometer at 100x magnification. The mean no. of tracheary elements per field was converted to tracheary elements per g f. wt of callus.

Each exp. was conducted twice with a minimum of 5 samples for each treatment within an expt.. Data from the temp. regime expt was analyzed using a 2-way analysis of variance. Data from the silver nitrate expt was analyzed using a 1-way analysis of variance. Within each expt specific treatments were compared using an *A priori* group comparison test (Sokal and Rohlf, 1969).

## RESULTS

Preliminary investigations showed that cotyledon-derived callus from both Wayne and Clark 63 cv. produced increased no. of tracheary elements following culture on a xylogenic medium supplemented with methionine in comparison to callus grown on a similar medium lacking this amino acid. It was further observed that soytean callus, similar to the results obtained with lettuce pith explants (Roberts and Baba, 1978), produced the greatest no. of tracheary elements on a medium containing 0.025  $\mu$ M methionine (Miller, unpublished results). The latter conc. of methionine was therefore used in all of the present expt..

### *Wayne*

Regardless of the incubation temp. at which the Wayne callus was grown, tracheary element differentiation was invariably stimulated with the addition of methionine to the xylogenic medium (Table 1). Within individual exp. this stimulation was significant for all treatments ( $P = 0.05$ ). It was unclear from the data that the temp. employed for callus initiation and maintenance (20°C vs. 25°C) had any influence on the differentiation occurring during the subsequent induction period. The lowest tracheary element counts, however, were recorded in exp. no. 1 with a 25°C initiation-maintenance temp. followed by a 20°C induction temp. (Table 1).

The addition of  $\text{AgNO}_3$  ( $20 \text{ mg l}^{-1}$ ) resulted in a 76.5% inhibition of xylem differentiation. In comparison to the no. of tracheary elements formed in callus on the B5D control medium, the combination of B5D +  $\text{AgNO}_3$  + methionine produced only a 40% inhibition of xylem differentiation (Table 2).

#### *Clark 63*

The addition of methionine to the medium had no significant effect on xylogenesis in the Clark 63 callus at an induction temp. of  $20^\circ\text{C}$ . At an induction temp. of  $25^\circ\text{C}$ , however, there was a significant ( $P = 0.05$ ) increase in xylem differentiation due to the exogenous methionine. Similar to the results with the Wayne, tracheary element formation was apparently independent of the callus initiation and maintenance temp.

The addition of  $\text{AgNO}_3$  to the B5D induction medium had relatively little effect (5.7% inhibition) on xylogenesis in the Clark 63 callus at  $25^\circ\text{C}$ . Although the Clark 63 callus on the B5D +  $\text{AgNO}_3$  + methionine medium produced 36% more tracheary elements than the callus on the B5D medium, the presence of  $\text{Ag}^+$  was inhibitory to the process because the callus on the B5D + methionine medium formed 102% more tracheary elements than similar callus samples on the B5D control medium (Table 2).

## DISCUSSION

The xylogenic response of the Wayne callus to exogenous methionine is similar to the observations by Roberts and Baba (1978) on the induction of xylem formation in explants of lettuce pith in the presence of traces of this amino acid. The present results support the view that the *in vitro* induction of xylem differentiation by auxin-cytokinin is stimulated by certain levels of endogenous ethylene. Presumably the Wayne callus produced 'xylogenic ethylene' at both  $20^\circ\text{C}$  and  $25^\circ\text{C}$  from the methionine substrate in the medium. Since organized tissues of Clark seedlings produce excessive amounts of ethylene at  $25^\circ\text{C}$  (Samimy and LaMotte, 1976), our results suggest that this temperature-dependent characteristic is also expressed by callus cells derived from cotyledon tissue taken from imbibed Clark 63 seeds. Exogenous methionine, however, was ineffective in stimulating

xylogenesis at 20°C in the cultured Clark 63 callus.

Earlier studies have shown that a  $\text{AgNO}_3$  conc. of approx.  $20 \text{ mg l}^{-1}$  was effective in blocking ethylene-regulated responses in *Pisum* seedlings (Beyer, 1978) and in *Nicotiana* leaf discs (Aharoni and Lieberman, 1979). Beyer (1978) demonstrated that the inhibitory effects of  $\text{Ag}^+$  were reversed by exogenous ethylene. Higher conc. of  $\text{AgNO}_3$ , e.g.,  $50\text{--}100 \text{ mg l}^{-1}$  caused tissue injury (Aharoni and Lieberman, 1979; Purvis, 1980). The inhibition of xylogenesis by  $\text{Ag}^+$  in the Wayne callus was slightly less in the presence of exogenous methionine (40% vs. 76.5%). The assumption can be made that this blockage reversal occurred because of competitive antagonism at the binding site of the hormone between  $\text{Ag}^+$  and the ethylene produced from the methionine. An alternative explanation may be advanced in order to explain the inhibitory effects of  $\text{Ag}^+$  on xylogenesis in the Clark 63 callus. The threshold level for ethylene to exert its stimulatory effect(s) on xylem differentiation may be higher in the Clark 63 tissue in comparison to the Wayne. Thus the relatively weak inhibitory effect of  $\text{Ag}^+$  in callus grown on the B5D +  $\text{AgNO}_3$  medium was because the level of endogenous ethylene was below the threshold level for stimulating xylogenesis. Inhibition by  $\text{Ag}^+$  was observed under cultural conditions that apparently involved higher endogenous ethylene conc., i.e., on the methionine-enriched media.

The relatively high variability in tracheary element cell counts was not unexpected, and this is an inherent problem associated with the quantitation of data obtained from callus cultures. Because of this variability in f. wt of callus, Snijman and Colleagues (1977) have expressed the view that callus cultures are unsuitable for studies of tracheary element differentiation. Regardless of the limitations, the present data does reveal certain trends, e.g., the stimulatory effects of methionine on xylogenesis as well as the blockage of the process by  $\text{Ag}^+$ . Further studies may provide an improved technique for the presentation of quantitative data from investigations of xylogenesis in callus.

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#### Legend to Table 1

<sup>a</sup>Each cell count given represents a mean value from a minimum of 5 samples. Each primary explant was first exposed to the callus initiation-maintenance temperature for a period of 24 d prior to 7 d of incubation at the induction temperature during xylogenesis. Methionine was employed at a conc. of 0.025  $\mu$ M.

<sup>b</sup>Tracheary element cell count is significant at  $p = 0.05$ .

#### Legend to Table 2

<sup>a</sup>Each cell count given represents a mean value from a minimum of 5 samples. Methionine and  $\text{AgNO}_3$  were employed at conc. of 0.025  $\mu$ M and 20  $\text{mg l}^{-1}$ , respectively. Following callus initiation (10 d) and two subcultures (14 d), the callus samples were incubated for 7 d on the xylogenic induction medium at a constant temp. of 25°C.

<sup>b</sup>Percentage change is significant at  $p = 0.05$ .

TABLE 1. Effects of temperature and exogenous methionine on the induction of xylogenes in callus explants of Wayne and Clark 63 soybean cultivars<sup>a</sup>

Expt No.	Callus initiation-maintenance temp. (°C)	Induction temp. (°C)	Tracheary elements ( $\times 10^4$ ) g <sup>-1</sup> f. wt			
			Wayne		Clark 63	
			B5D	B5D + methionine	B5D	B5D + methionine
1	20	20	6.45	13.00 <sup>b</sup>	6.81	4.13
1	20	25	8.46	14.39 <sup>b</sup>	5.04	8.98 <sup>b</sup>
2	20	20	5.58	18.67 <sup>b</sup>	1.63	1.02
2	20	25	5.61	14.16 <sup>b</sup>	1.71	5.39 <sup>b</sup>
1	25	20	2.62	7.91 <sup>b</sup>	4.10	3.01
1	25	25	5.42	9.39 <sup>b</sup>	3.55	11.44 <sup>b</sup>
2	25	20	15.91	18.97 <sup>b</sup>	0.33	0.41
2	25	25	13.84	22.48 <sup>b</sup>	2.81	4.09 <sup>b</sup>

TABLE 2. Inhibitory effects of AgNO<sub>3</sub> on xylogenes in callus explants of Wayne and Clark 63 soybean cultivars<sup>a</sup>

Induction medium	Wayne		Clark 63	
	Tracheary elements ( $\times 10^4$ ) g <sup>-1</sup> f. wt	% change from B5D control	Tracheary elements ( $\times 10^4$ ) g <sup>-1</sup> f. wt	% change from B5D control
B5D	2.43	—	1.06	—
B5D + AgNO <sub>3</sub>	0.57	76.5 inhibition <sup>b</sup>	1.00	6.0 inhibition
B5D + meth.	4.55	87.0 stimulation <sup>b</sup>	2.14	102.0 stimulation <sup>b</sup>
B5D + AgNO <sub>3</sub> + meth.	1.46	40.0 inhibition <sup>b</sup>	1.44	36.0 stimulation <sup>b</sup>