Exogenous Methionine as a Nutrient Supplement for the Induction of Xylogenesis In Vitro

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ABSTRACT

Induced xylogensis in explants of lettuce pith parenchyma was greatly influenced by the presence of exogenous methionine (0.1 to 0.025 μM) in the culture medium. At the various concentrations of methionine tested, tracheary element cytodifferentiation was greatly stimulated in the majority of explants. Differentiation, however, was markedly depressed in a small number of explants grown under the same cultural conditions in the presence of methionine. Cytodifferentiation in control explants, cultured on a similar medium lacking methionine, gave consistent tracheary cell counts with relatively little variability. These data suggest that endogenous ethylene may play an important role in the induction of tracheary element differentiation. The internal concentration of ethylene produced within the cultured tissue may be a critical factor in determining whether the hormone will stimulate or suppress the initiation of cytodifferention. Some thick-walled and pitted cells were observed, and these may represent partially-differentiated xylem elements.

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Introduction

Methionine is a major precursor for the biosynthesis of ethylene in higher plant tissues (Abeles, 1972, 1973; Yang, 1974). The incorporation of methionine into a culture medium supporting the growth of internode segments excised from a short-day plant, *Plumbago indica* L. var. Angko, induced the formation of flower buds under noninductive photoperiods. The treatment of similar cultures with exogenous ethylene produced the same results (Nitsch, 1968). Sakai and Imaseki (1972) demonstrated that exogenous methionine acts as an *in vivo* precursor of ethylene biosynthesis in auxin-treated mung bean hypocotyl segments, and other workers have shown that ethylene production in apple tissue slices is greatly stimulated by exogenous methionine (Liberman, Kunishi, Mapson and Wardale, 1966). These results suggest that methionine may be employed in an external medium for the enhancement of ethylene production by isolated plant tissues.

Definitive evidence is lacking to support the hypothesis that ethylene acts as a functional hormone during the initiation of tracheary element differentiation (see review by Roberts, 1976). Nevertheless, several workers have provided evidence that ethylene may influence either growth, cytodifferentiation, or lignification. The application of mechanical stress to branches of various trees resulted in significant increases in the ethylene content of the internal atmospheres of the stressed branches (Leopold, Brown and Emerson, 1972), and reaction wood formation may be an expression of stress-induced ethylene production. Trauma-induced ethylene biosynthesis has been demonstrated in secondary xylem tissue (Cooper, 1972). The production of ethylene by the secondary xylem of *Pinus radiata* has been associated with heartwood formation (Shain and Hillis, 1973), and phenolic acid and lignin biosynthesis has been stimulated by ethylene in swede root tissue (Rhodes and Wooltorton, 1973). The application of exogenous ethylene gas to the trunks of *Pinus radiata* and *Liquidambar styraciflua* resulted in the production of greater amounts of secondary xylem

than did untreated controls (Neel, 1970). Increased xylogenesis in stem-wounded *Coleus* shoots subjected to geotropic stimulation on a clinostat was probably related to stress-induced ethylene biosynthesis (Roberts and Fosket, 1962). The best evidence for a role of ethylene in secondary xylem differentiation was presented by Zobel (1974) in connection with his discovery of the tomato mutant, *diageotropica* (*dgt*), which is deficient in the capacity to synthesize sufficient quantities of ethylene for normal development. In comparison with the stem of a normal tomato plant, the *dgt* mutant produced a significantly greater number of tracheids. The xylem tissue of the *dgt* mutant completely lacked vessels, whereas large vessels were conspicuous in the normal tomato stem (Zobel, 1974).

The current study involves the addition of trace amounts of L-methionine to a culture medium prepared for the induction of the cytodifferentiation of tracheary elements in explants of lettuce pith parenchyma (Banko, Roberts and Boe, 1976). The assumption was made that ethylene biosynthesis by the explants will be enhanced following the uptake of the methionine from the medium. A preliminary examination was made of the qualitative and quantitative responses of the explants to the cytodifferentiation of tracheary elements.

Materials and Methods

Tissue explants were prepared from the trimmed core of pith removed from heads of Romaine lettuce (*Lactuca sativa* L. cv. Româna) as described previously (Dalessandro and Roberts, 1971). The trimmed central core of the lettuce head was surface sterilized in a 10 per cent aqueous solution of Chlorox for 10 min. The core was rinsed three times with sterile double-distilled water, and cylinders of pith parenchyma were aseptically excised with a 5-mm I. D. sterile cork borer. The cylinders of tissue were cut into explants 3 mm thick and rinsed twice with sterile doubledistilled water. The explants were lightly blotted on sterile Whatman No. 1 filter paper and transferred singly to Kimax culture tubes (16 mm O. D. x 75 mm) containing

approximately 5 ml of a xylogenic culture medium. The medium consisted of a Murashige and Skoog basal medium (1962) supplemented with 1 per cent Bacto-Agar, 3 per cent (w/v) sucrose, *myo*-inositol (100 mg/l), thiamin·HCl (0.1 mg/l), glycine (2.0 mg/l), nicotinic acid (0.5 mg/l), pyrodoxin·HCl (0.5 mg/l), indoleacetic acid (10.0 mg/l), and kinetin (0.2 mg/l. L-Methionine[1] was incorporated into the medium in concentrations varying from 10.0 μM to 0.025 μM. The open ends of the culture tubes were enclosed with a sterile wrap of aluminum foil. The tubes were placed upright in storage jars (100 mm x 80 mm; 10 tubes per jar), and the jars were wrapped with amluinum foil to exclude light. The culture tubes were maintained at 25 °C during the 7 day incubation period. At the conclusion of each experiment tracheary elements were examined microscopically for anatomical variations in some explants, and other explants were macerated for tracheary cell counts. For the microscopic examination of individual tracheary elements the explants were cleared with 4 per cent NaOH at 55 °C for about 12 h. The NaOH was decanted and the explants were stained with a 0.03 per cent aqueous solution of safranin 0 for 30 min. at 55 °C. This was followed by destaining with several changes of 1.0 N HCl over a 2 h period. After destaining the explants were stored in glycerol for subsequent examination. Tracheid numbers were determined by a modification of the Brown and Rickless (1949) maceration technique. The explants were removed from the culture tubes and placed singly in 6-ml shell vials. The explants were immersed in approximately 1 ml of maceration fluid consisting of equal parts of 5 per cent HCl and 5 per cent chromic acid. After maceration for about 24 h at room temperature, the maceration fluid was gently removed with a hypodermic syringe and replaced with an equivalent amount of distilled water. The macerated explant in the distilled water was drawn repeatedly into a 2.0 ml

1) L-Methionine was purchased from the Sigma Chemical Company, P. O. Box 14508, St. Louis, Missouri 63178, U. S. A.

hypodermic syringe fitted with a No. 22 guage needle. After a thorough homogenation the sample was transferred to a volumetric tube calibrated to contain 2.0 ml. The original shell vial was flushed repeatedly with distilled water, and the washings were transferred to the volumetric tube. Distilled water was added to the volumetric tube for a final sample volume of 2.0 ml. One ml of the sample was transferred by hypodermic syringe to a 25 mm x 40 mm x 1 mm Sedgewick-Rafter plankton-counting chamber (Klein and Klein, 1970). Tracheary elements were counted in 10 optical fields with the aid of a Whipple eyepiece micrometer at a magnification of 100x. The total number of tracheary elements in each individual explant was calculated (Banko, Roberts and Boe, 1976).

Results

The incorporation of trace amounts of methionine (0.05 to 0.025 μM) to the culture medium was highly effective in stimulating the induction of xylogenesis in the majority of the explants of a given experiment (Fig. 1). In preliminary trials concentrations higher than 0.05 μM were considerably less effective in stimulating xylem differentiation, and the inhibitory effects observed in some explants were increased. For example, counts of approximately 100,000 tracheids

Fig. 1. Tracheary Element Differentiation in Lettuce Pith Explants Cultured on Methionine-Containing Media[1)]

Methionine Concentration (μM)	Tracheary Elements (x 10^4) Experiment No. 1	Experiment No. 2
0.0	3.69±0.43 (8)	3.55±0.70 (9)
10.0	4.24±0.93 (6)	
1.0	4.65±1.17 (7)	
0.1	5.77±1.38 (7)	
0.05	6.65±1.82 (10)	5.89±1.83 (10)
0.025	8.48±1.35 (10)	7.27±1.28 (8)

1) The mean number of tracheary elements is given with the standard error of the mean for each concentration of methionine tested. The number given in parentheses following the standard error indicates the total number of explants that were macerated and counted for each treatment.

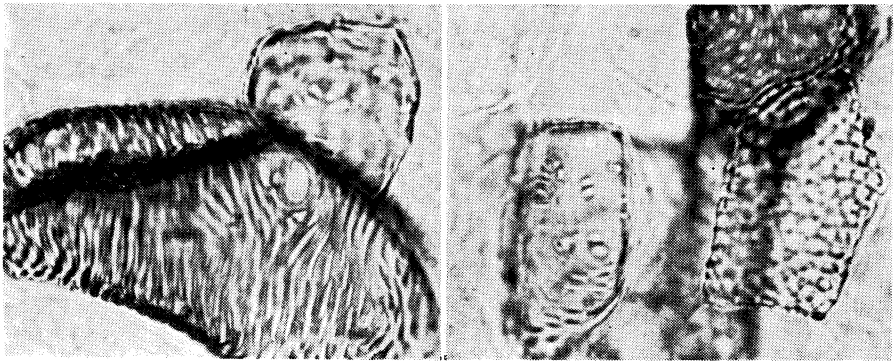
Fig. 2. Selected Data of Tracheary Element Counts from Individual Explants Receiving Various Concentrations of Methionine[1)]

Explant No.	10.0 μM (Exp. 1)	0.05 μM (Exp. 2)	0.025 μM (Exp. 1)
1	5.50	9.96	10.54
2	4.87	7.69	10.05
3	4.85	6.87	9.97
4	4.29	6.34	9.24
5	3.33	5.03	8.42
6	2.64	4.94	7.86
7		4.81	7.56
8		4.48	7.26
9		4.38	7.15
10		4.37	6.94
Means	4.24	5.89	8.48

1) The cell counts are given x10^4. For purposes of comparison control explants, without exogenous methionine, contained a mean number of tracheids in Exp. 1 of 3.69 x 10^4 and 3.55 x 10^4 in Exp. 2.

per explant weer found at concentrations of 0.025 to 0.05 μM methionine, whereas the maximum number of tracheids observed at a concentration of 10.0 μM was about one-half of this value (Fig. 2). There was no apparent difference in the extent of the callus production by the explants cultured either in the presence or absence of methionine, and there were no signs of organogenesis in any of the explants at all concentrations of methionine tested. Although a careful histological study has not been made of the arrangement of the newly-formed tracheids of the methionine-treated explants, cleared and stained whole explants revealed a greater number of tracheids in the central region of the explant than has been described previously for explants cultured in the absence of methionine (Dalessandro and Roberts, 1971). In addition, extremely large numbers of tracheids were observed in the callus surrounding the initial explant. Most of the tracheary elements displayed the typical scalarifom-reticulate secondary wall thickenings. There were, however, several large hickwalled and pitted cells that may represent partially-differentiated xylem elements (Fig. 3—4). In some of these unique cells bar-like secondary wall

Fig. 3—4. Tracheary elements differentiated in the presence of exogenous methionine. Fig. 3. Spherical cell, contiguous with a normally-differentiated tracheary element, exhibits an irregular pit pattern. x 200. Fig. 4. Cluster of tracheary elements possessing various patterns of secondary wall thickening. Note weakly-differentiated cell in upper part of photomicrograph in which secondary wall striations are barely discernible and are apparent in only certain regions of the cell. x230.



thickenings were barely discernible, and these cells were always located contiguous with fully-formed tracheids. These unusual cells have been described previously in pith parenchyma explants of lettuce (Cawthon, 1972) and in tobacco callus (Snijman, 1972) following growth of the cultured tissues in the presence of gibberellic acid.

Discussion

The remarkable stimulation of xylogenesis, resulting from the assimilation of exogenous methionine by the parenchymatous explants, is undoubtedly a hormonal response to certain critical levels of endogenous ethylene. This is the first report that ethylene, in addition to auxin and cytokinin, acts as a hormonal 'critical variable' (Roberts,

1976) in the initiation of xylogenesis. Methionine is an important amino acid in the initiation of protein synthesis (see review, Zalik and Jones, 1973), and this could be a factor in the cytodifferentiation requirement. It is difficult to experimentally determine the concentrations of endogenous ethylene effective in xylogenesis. Ideally a quantitative relationship should be shown between methionine levels, ethylene production, and xylem formation. Unfortunately, the culture vessel must be sealed for chromatographic analysis, and this rapidly leads to ethylene levels that are toxic to both cell division and cytodifferentiation (Zobel and Roberts, 1975). In fact, it is doubtful if the measurement of atmospheric ethylene within the vessel would be meaningful. Ethylene formed within the tissue, either by the differentiating cells themselves or by neighboring cells, has some immediate effect on the initiation of the cytodifferentiation process prior to release to the external air. In our experiments as little as 10.0 μM of exogenous methionine showed some toxicity to cytodifferentiation, and previous workers may have employed too high concentrations in order to attempt to demonstrate an ethylene response (Rubinstein and Leopold, 1962).

The significance of the poorly-developed secondary wall striations in some of the differentiated cells is subject to speculation. Since these cells are apparently produced in the presence of either gibberellic acid or ethylene, it is possible that these cells reflect some hormone-induced malfunction of the microtubules associated with the orientation of the secondary wall microfibrils. Brower and Hepler (1976) have reviewed the current status of the role of microtubules on secondary wall deposition in xylem elements, but our knowledge on the hormonal regulation of this process is still meager. Gibberellins may be involved with microtubule function (Shibaoka, 1972, 1974; Fragata, 1970), and the well-known radial cell enlargement response to ethylene must involve a reorientation of the structural components of the cell wall.

The high variability in the xylogenic response by the methionine-treated explants may reflect differences between individual explants

in the capability for ethylene production due to some limiting factor in the biosynthetic pathway. Ethylene output is complicated because it represents a summation of the biosynthesis induced by (a) the initial wound response, (b) auxin, (c) methionine, and (d) sucrose and other carbohydrates. Since sucrose stimulates ethylene biosynthesis (Moore, 1976), the induction of xylogenesis by exogenous sucrose (DeMaggio, 1972) may involve the sugar-induced production of xylogenic levels of etylene. The biosynthesis of wound-induced ethylene often involves methionine (Hanson and Kende, 1976), and a more consistent cytodifferentiation response to ethylene might be achieved by transferring cultured explants from a minus-methionine medium to a methionine-containing medium following the initial flush of ethylene associated with excision of the fresh tissue.

Gamborg and LaRue (1971) reported that cell suspension cultures of *Ruta* sp. were capable of cytodifferentiation, while similar cultures of *Rosa* always remained undifferentiated. The addition of 2, 4-D enhanced ethylene production in the *Ruta* cultures, but not in the rose cells. The authors suggested that there may be some connection between auxin-induced ethylene biosynthesis and the capability for cytodifferentiation. It would be of interest to determine the relative effectiveness of 2,4-D and NAA, compared to IAA, in the initiation of cytodifferentiation in the presence of methionine.

We have conducted some preliminary experiments that support the view that ethylene influences xylogenesis (L. W. Roberts and C. E. Russell, unpublished). Lettuce pith explants, incubated on a xylogenic medium (without methionine) in culture tubes, were capped with a single layer of Parafilm M[1] instead of aluminum foil. Gas chromato-

1) Parafilm M, manufactured by the American Can Company, is a commercial sealant composed of a rubber-wax-polymer combination, and the sample employed in our study had a thickness of approximately 4.8 mil (122 μ). We are grateful to R. F. De Long, American Can Company, for his technical assistance.

graphic analysis of the ethylene remaining within the atmosphere of the culture vessels indicated concentrations fluctuating during the 7 day incubation period between 1-2 μl/l. Xylogenesis under these conditions was similar to our results obtained with methionine, i. e., there was a high variability in the numbers of tracheary elements formed in individual explants. Some explants revealed relatively high numbers of tracheids, whereas in other explants the cytodifferentiation process was sharply inhibited. The range of cell counts resulting from the Parafilm M experiments more closely approximated methionine concentrations of 10.0 μM and above than the lesser concentrations. Concentrations of considerably less than 1 μl/l are probably required for the optimal stimulation of xylogenesis, and this estimate is conservative since Zobel (1974) found that 0.005 μl/l ethylene normalized *dgt* mutant characteristics.

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