Plant Cell, Tissue and Organ Culture **25**: 45–48, 1991.

Stability and utilization of picloram, vitamins, and sucrose in a tissue culture medium

Steven R. Hagen, Paul Muneta, Jorg Augustin & Duane LeTourneau
Food Research Center, University of Idaho, Moscow, ID 83843, USA

Received 21 August 1990; accepted in revised form 3 December 1990

Key words: Murashige & Skoog medium, nicotinic acid, picloram, pyridoxine, sucrose, thiamine

Abstract

Nicotinic acid, pyridoxine, and picloram were stable in a liquid MS culture medium (pH 5.5–5.6) during autoclaving and during cell-free incubation in the dark at 5°C or 25°C for up to 6 weeks. Thiamine loss under the same conditions was 16% at 5°C and 18% at 25°C. Five percent of the sucrose in the liquid medium was hydrolyzed during autoclaving. During cell-free incubation in the light (100 μE m^{-2} s^{-1}) at 25°C, pyridoxine was not detected after 6 days, while 78% of the picloram and 56% of the thiamine were degraded after 6 weeks. All of the niacin and pyridoxine, 13% of the picloram and 42% of the thiamine in a liquid MS culture medium were utilized in 4 days by potato (cv. Lemhi Russet) tuber suspension cultures growing in the dark at 25°C.

Abbreviations: B5–Gamborg et al. medium (1968), 2,4–D 2,4–dichlorophenoxyacetic acid, IAA–indoleacetic acid, MS–Murashige & Skoog (1962), NAA–naphthaleneacetic acid, PAA–phenylacetic acid

Introduction

Plant tissue culture media contain a variety of organic constituents whose concentrations are varied depending on the tissue being cultured (George et al. 1987). While the formulations of these media have been studied extensively, little research has been done to assess the changes in media during preparation, storage and incubation with cells (Dunlap et al. 1986).

The stability of auxins in cell-free liquid media has been studied to a limited extent. Yamakawa et al. (1979) found that IAA and 2,4-D in MS salts with sucrose were not decomposed by autoclaving, shaking, and oxygenation in the dark. However, upon exposure to light IAA decomposed, while 2,4-D was unaffected (Yamakawa et al. 1979). Similarly, Dunlap et al. (1986) observed that NAA and 2,4-D levels remained constant in media containing 0-100% MS salts stored in the dark at 30°C for up to 35 days. Contrary to the results of Yamakawa et al. (1979), IAA levels decreased as MS salt concentrations increased (Dunlap et al. 1986). In further experiments, IAA loss was found to be accelerated by media pH levels below 7, in the presence of iron and nitrates, and in light (Dunlap & Robacker 1988). PAA in MS and B5 media was stable to autoclaving and storage in the dark at 5°C for up to 28 days, however, PAA levels dropped by up to 15% in the MS medium after 28 days at room temperature (Leuba et al. 1989).

Picloram is a potent auxin useful for the initiation and maintenance of callus and suspension cultures from certain plant tissues (Kefford & Caso 1966; Huang & Chi 1988; Hagen et al. 1990). While the stability of some auxins during cell-free incubation has been investigated, there have been no similar tests with picloram. In addition, there are no reports on the stability of other organic media components (e.g., vitamins, sucrose) during cell-free incubation, or on the utilization of these components by growing cells.

Research Paper No. 90516 of the Idaho Agricultural Experiment Station.

As part of a comparative study of potato tuber carbohydrate metabolism, we have used callus derived from tubers of several cultivars for use as a model system (Muneta et al. 1990; Hagen et al. 1991). Experiments were conducted to measure the stability and utilization of picloram, vitamins, and sucrose in the tissue culture medium used for callus subculture. The concentrations of these components were measured in medium exposed to various incubation regimes in the presence and absence of growing cells.

Materials and methods

The liquid medium used for these experiments contained the MS (Murashige & Skoog 1962) inorganic salts and inositol, the B5 vitamin mixture of Gamborg et al. (1968), 3% (w/v) sucrose, and 10 μM picloram (4-amino-3,5,6-trichloropicolinic acid). For each experiment, 50 ml of medium was dispensed into 250 ml Erlenmeyer flasks (Pyrex No. 4442). After autoclaving at 1.1 kg cm^{-2} for 15 min, the pH of the medium was 5.5–5.6.

The culture medium was tested for changes in nicotinic acid, pyridoxine, thiamine, picloram, and sugar concentrations after various incubation treatments, in the absence and presence of potato (cv. Lemhi Russet) tuber suspension cultures (0.5 ml subculture volume). For each experiment, cell-free medium was analyzed before and after autoclaving. For the cell-free experiments, flasks containing medium were incubated in the dark at 5°C or 25°C, or at 25°C under fluorescent lights (100 μE m^{-2} sec^{-1}) on a 16 h light/8 h dark photoperiod. Flasks for the suspension culture experiments were incubated in the dark at 25°C on a gyratory shaker (125 rpm). Controls without cells, including an additional 0.5 ml of HPLC grade water, were incubated under the same conditions. Potato tuber suspension cultures were initiated from callus and subcultured as previously described (Hagen et al. 1990).

Nicotinic acid, pyridoxine, thiamine and picloram were determined by HPLC. Aliquots of cell-free medium were analyzed after correction of volume loss by addition of HPLC grade water. The medium containing cells was filtered by vacuum through a scintered glass funnel (Pyrex No. 36060), and volume loss was corrected by adding HPLC grade water. The eluant consisted of 800 ml of an aqueous phase (10 ml per liter HPLC grade glacial acetic acid, 1 g per liter each of anhydrous 1-pentane and 1-hexane sulfonic acid sodium salts) mixed with 200 ml of HPLC grade methanol. Filtered (Gelman nylon Acrodiscs, 0.2 μm) aliquots were applied with an autoinjector (Waters 712B), and eluted isocratically (Waters M6000A) at 1.5 ml min^{-1} through a Waters micro-Bondapack C18 10 μm particle size RCM 8 × 10 column. The analytes were detected spectrophotometrically (Waters M440) (280 nm, 0.05 AUFS for pyridoxine; 254 nm, 0.05 AUFS for the other vitamins and picloram). Peak purities were determined by comparing the ratio of absorbances at 254 and 280 nm for pure calibration standards to corresponding peaks in the sample chromatograms. A computerized data station (Waters Maxima 820) was used to quantify the data. Reducing and non-reducing sugars were determined colorimetrically with the Folin-Wu procedure (AOAC 1975). For dry weight determination, cells from each flask were dried to a constant weight in a vacuum oven at 60°C.

Results and discussion

The results of the cell-free medium experiment are summarized in Table 1. Autoclaving did not lower the vitamin or picloram content. Pyridoxine and picloram levels did not change after incubation in the dark for up to 6 weeks at 5°C or 25°C. Thiamine levels were significantly lower than pre-autoclaving levels after 4 and 6 weeks in the dark at 5°C and 25°C. The nicotinic acid concentration was not altered by any treatment through the 2 week sampling period. Quantitation of nicotinic acid in the 4 and 6 week samples was not possible due to the presence of co-eluting compounds during the HPLC analysis. Pyridoxine and picloram were rapidly destroyed when the medium was illuminated (100 μE m^{-2} sec^{-1}). Pyridoxine was completely destroyed after 1 week in the light at 25°C, and nearly 80% of the picloram was destroyed by the end of the experiment. In a limited follow-up experiment, pyridoxine was not detected after 6 days in the same incubation conditions. Thiamine concentrations were significantly lower in the light vs. dark treatments after 4 weeks. This experiment was repeated with comparable results.

Table 1. Percentage of vitamins and picloram remaining in a cell-free liquid culture medium after autoclaving and various incubation treatments.

Treatment	Nicotinic acid	Pyridoxine	Picloram	Thiamine
Pre autoclave	100.0^{c}	100.0^{a}	100.0^{a}	100.0^{a}
Post autoclave	100.4^{c}	99.4^{a}	102.5^{a}	91.8^{ab}
5°C Dark, 2 wk	110.6^{bc}	96.4^{a}	101.3^{a}	90.6^{ab}
5°C Dark, 4 wk	NA[1]	95.8^{a}	94.6^{ab}	87.6^{b}
5°C Dark, 6 wk	NA	94.2^{a}	94.0^{ab}	84.3^{b}
25°C Dark, 2 wk	111.7^{bc}	93.0^{a}	99.2^{a}	91.0^{ab}
25°C Dark, 4 wk	NA	96.8^{a}	94.4^{ab}	83.5^{b}
25°C Dark, 6 wk	NA	93.2^{a}	98.7^{a}	81.8^{b}
25°C Light, 1 wk	103.6^{c}	0.0^{b}	78.2^{bc}	87.4^{b}
25°C Light, 2 wk	113.9^{abc}	0.0^{b}	64.3^{c}	82.2^{b}
25°C Light, 4 wk	NA	0.0^{b}	39.9^{d}	54.6^{c}
25°C Light, 6 wk	NA	0.0^{b}	21.7^{e}	44.3^{c}

[1] Nicotinic acid quantitation was not possible in some cases due to interferences in the chromatographic separation.
Means in the same column followed by the same letter are not significantly different ($P < 0.05$; Least Significant Difference test). There were three samples per treatment.

Table 2. Percentage of vitamins and picloram remaining in a liquid culture medium after autoclaving and incubation for 4, 6, or 8 days in the dark at 25°C on a gyratory shaker (125 rpm).

Treatment	Nicotinic acid	Pyridoxine	Picloram	Thiamine
Pre autoclave	100.0^{a}	100.0^{b}	100.0^{ab}	100.0^{a}
Post autoclave	97.9^{a}	98.2^{b}	102.9^{a}	102.0^{a}
4 d, no cells	101.7^{a}	105.5^{a}	102.9^{a}	104.6^{a}
6 d, no cells	99.0^{a}	106.4^{a}	96.0^{ab}	97.5^{a}
8 d, no cells	99.4^{a}	101.6^{b}	96.8^{ab}	99.0^{a}
4 d, cells[1] (0.08)[2]	0.0^{b}	0.0^{c}	87.2^{bc}	57.7^{b}
6 d, cells (0.24)	0.0^{b}	0.0^{c}	76.8^{c}	0.0^{c}
8 d, cells (0.44)	0.0^{b}	0.0^{c}	60.3^{d}	0.0^{c}

[1] Potato tuber suspension cultures from cv. Lemhi Russet.
[2] Final mean dry weight (g) of cells (initial subculture dry weight was 0.04 g).
Means in the same column followed by the same letter are not significantly different ($P < 0.05$; Least Significant Difference test). There were three samples per treatment.

Table 3. Reducing, non-reducing, and total sugar content (mg ml^{-1} medium) in a liquid culture medium after autoclaving and incubation for 4, 6, or 8 days in the dark at 25°C on a gyratory shaker (125 rpm).

Treatment	Reducing	Non-Reducing	Total
Pre autoclave	0.00^{d}	33.06^{a}	33.06^{a}
Post autoclave	1.65^{cd}	31.45^{a}	33.10^{a}
4 d, no cells	1.65^{cd}	31.50^{a}	33.15^{a}
6 d, no cells	1.65^{cd}	31.79^{a}	33.44^{a}
8 d, no cells	1.63^{cd}	31.73^{a}	33.37^{a}
4 d, cells[1] (0.08)[2]	2.87^{c}	17.56^{b}	20.43^{b}
6 d, cells (0.24)	9.86^{b}	12.40^{b}	22.26^{b}
8 d, cells (0.44)	14.22^{a}	5.38^{c}	19.60^{b}

[1] Potato tuber suspension cultures from cv. Lemhi Russet.
[2] Final mean dry weight (g) of cells (initial subculture dry weight was 0.04 g).
Means in the same column followed by the same letter are not significantly different ($P < 0.05$; Least Significant Difference test). There were three samples per treatment.

The utilization of vitamins and picloram by actively growing cells is shown in Table 2. Over the 8 day period the cells increased about 10-fold in dry weight. There were no significant losses of vitamins or picloram in the control (cell free) treatments. However, there were dramatic losses of vitamins and picloram when cells were present. Nicotinic acid and pyridoxine were completely removed from the medium in 4 days, while about 40% of the picloram was utilized in 8 days, and all of the thiamine was utilized in 6 days.

Table 3 summarizes the changes in reducing (glucose and fructose) and non-reducing (sucrose) sugars in the suspension culture experiment. Autoclaving hydrolyzed 5% of the sucrose into glucose and fructose. There were no other changes detected in the control treatments. The actively growing callus hydrolyzed some of the sucrose, and some was utilized for growth. This experiment was repeated with comparable results.

It is clear from the results that illumination destroyed pyridoxine in the MS medium very rapidly, and thiamine at a slower rate. Picloram was also susceptible to breakdown in light, as are several other auxins commonly used in media (Yamakawa et al. 1979, Dunlap et al. 1986; Dunlap & Robacker 1988; Leuba et al. 1989). The results also showed that nicotinic acid, pyridoxine, picloram, and thiamine are rapidly utilized by growing cells. The utilization of organic components in liquid and solid media by cells during incubation, and non-biological losses during preparation and storage need to be studied further in order to optimize media composition for cell culture.

References

Assoc. Offic. Anal. Chem. (1975) Official Methods of Analysis. Twelfth Ed. Sections 31:054–31.055

Dunlap JR, Kresovich S & McGee RE (1986) The effect of salt concentration on auxin stability in culture media. Plant Physiol. 81: 934–936

Dunlap JR & Robacker KM (1988) Nutrient salts promote light-induced degradation of indole-3-acetic acid in tissue culture media. Plant Physiol. 88: 379–381

Gamborg OL, Miller RA & Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Cell Res. 50: 151–158

George EF, Puttock DLM & George HJ (1987) Plant Culture Media, Volume 1. Formulations and Uses. Exegetics, Westbury, England

Hagen SR, LeTourneau D, Muneta P & Brown J (1990) Initiation and culture of potato tuber callus tissue with picloram. Plant Growth Regul. 9: 341–345.

Hagen SR, Muneta P, LeTourneau D & Brown J (1991) Effect of temperature on the starch content of potato callus tissue. Am. Potato J. In press

Huang L-C & Chi D-L (1988) Pivotal roles of picloram and gelrite in banana callus culture. Environ. Exp. Bot. 28: 249–258

Kefford NP & Caso OH (1966) A potent auxin with unique chemical structure--4-amino-3,5,6-trichloropicolinic acid. Bot. Gaz. 127: 159–163

Leuba V, LeTourneau D & Oliver D (1989) Stability of phenylacetic acid in liquid media. J. Plant Growth Regul. 8: 163–165

Muneta P, Hagen S, LeTourneau D & Brown J (1990) Effect of temperature on the sugar content of potato callus tissue. Am. Potato J. 67: 209–215

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Yamakawa T, Kurahashi O, Ishida K, Kato S, Kodama T & Minoda Y (1979) Stability of indole-3-acetic acid to autoclaving, aeration and light illumination. Agric. Biol. Chem. 43: 879–880