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The Culture of Plant Cells with Ammonium Salts as the Sole Nitrogen Source

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OLUF L. GAMBORG AND J. P. SHYLUK
Prairie Regional Laboratory, National Research Council of Canada, Saskatoon, Saskatchewan, Canada

ABSTRACT

Soybean cell suspension cultures grew on defined media with ammonium as the sole nitrogen source if Krebs cycle acids were added. Satisfactory growth was obtained with ammonium salts of citrate, malate, fumarate, or succinate, when compared with the regular medium containing nitrate and ammonium. Little or no growth occurred when ammonium salts of shikimate, tartrate, acetate, carbonate, or sulfate were used. The cells also grew well with L-glutamine as nitrogen source. The specific activities of glutamine synthetase and isocitrate dehydrogenase (nicotinamide adenine dinucleotide phosphate) were lower than in cells grown on a nitrate medium, but ammonium enhanced the activity of glutamate dehydrogenase. Cells of soybean, wheat, and flax have been cultured for an extended period on the ammonium citrate medium.

The most suitable inorganic nitrogen source for growing plant cells is a mixture of ammonium and nitrate (2, 3, 7). Plant cells in culture do not grow readily on ammonium salts alone although ammonium is a more direct nitrogen source than nitrate for protein production. Filner (3) could not grow tobacco cells on ammonium salts. Rice callus did not grow on agar media containing ammonium sulfate but grew on ammonium citrate (13).

We observed that soybean cells, grown in a liquid, defined medium, needed ammonium in order to utilize nitrate (7). Ammonium was most effective at concentrations of 2 to 4 mM. At higher or lower concentrations the cell yields decreased. Glutamine, but not other amino acids or asparagine, could replace ammonium (5).

The cell cultures provided a convenient system to determine the effect of ammonium on enzyme activity in the cells. The concentrations of glutamine synthetase (EC 6.3.1.2), glutamate dehydrogenase (EC 1.4.1.2), and isocitrate dehydrogenase (EC 1.1.1.42) were determined. This paper reports that soybean cells will grow with ammonium salts as the sole nitrogen source if certain Krebs cycle acids are added. Ammonium enhanced glutamate dehydrogenase activity and reduced the levels of glutamine synthetase and isocitrate dehydrogenase.

MATERIAL AND METHODS

Culture Conditions. The soybean cells were cultured in De Long flasks in liquid B5 medium as described earlier (4, 6, 7). This medium contains mineral salts, sucrose, the B-vitamins, and 2,4-dichlorophenoxyacetic acid (1 mg/liter). Nitrogen was supplied as 25 mM potassium nitrate and 1 mM ammonium sulfate. The pH was 5.5 which is optimal for growth (7). The cell inoculum was washed in fresh medium which contained no nitrate or ammonium before being added to the culture flasks. The cells used for inoculum were grown in the B5 medium but grew immediately when transferred to a suitable ammonium medium without requiring a period of adaptation. The carboxylic acids were filter-sterilized and neutralized with ammonium hydroxide and potassium hydroxide.

The cells were usually harvested after 5 or 6 days by filtration and then washed with water. Dry weights were determined after drying aliquots of cells in a vacuum oven at 60 C for 18 hr. Total protein was estimated by the micro-Kjeldahl method.

Enzyme Extraction. The cells were collected by filtration on Miracloth and washed. A weighed amount of cells was mixed with 1-mm glass beads and 50 mM Tricine (*N*-tris(hydroxymethyl)methylglycine) buffer, pH 7.5, containing 1 mM 2-mercaptoethanol, in the proportions 1:1·5:1 (w/w/v) in a 75-ml homogenizer flask and homogenized for 90 sec at 2 to 4 C in a Braun homogenizer, model MSK. The homogenate was separated from the beads by decantation. A measured aliquot of the homogenate was added to 2 volumes of ethanol at −20 C, and the precipitate was used for total protein analysis. The remainder of the homogenate was centrifuged, and the supernatant was lyophilized and stored. The freeze-dried preparations were suspended in water, centrifuged, and passed through a column of Sephadex G-50, and the protein fraction was immediately assayed for enzyme activity.

Enzyme Assays. Isocitrate dehydrogenase, shikimate dehydrogenase, and glutamate dehydrogenase were determined by recording the change in absorption of the pyridine nucleotides at 340 nm on a Gilford automatic spectrophotometer in the presence of the appropriate substrate. The formation of γ-glutamylhydroxamate was used to measure glutamine synthetase (12).

One unit of enzyme is the amount required to produce 1 μmole/min at 30 C.

Assays Conditions

Isocitrate Dehydrogenase. The reaction mixture consisted of 5 μmoles of $MnCl_2$; 5 μmoles of DL-alloisocitrate; 0.2 μmole of NADP or NAD; 50 μmoles of Tricine buffer, pH 7.5; and enzyme preparation in a total volume of 1.0 ml.

Glutamate Dehydrogenase. The reaction mixture had the following composition: 20 μmoles of ammonium sulfate; 10 μmoles of α-ketoglutarate; 0.1 μmole of NADH; 50 μmoles of Tricine buffer, pH 8.0; and enzyme in a total volume of 1.0 ml.

Shikimate Dehydrogenase. The reaction mixture consisted of

Table I. *Variation in Yields of Soybean Cells Grown on Different Sources of Nitrogen in Suspension Culture*

The inoculum was cells grown in the B5 medium and washed in nitrogen-free medium. Inoculum was 23 mg, growing period 6 days, and culture volume 40 ml in 250-ml flasks.

Source of Reduced Nitrogen	Concn	KNO_3	Final Dry Weight
	mM	25 *mM*	*mg*
None	—	+	74
Ammonium sulfate (B5)	1	+	388
L-Glutamine	4	+	301
D + L-Glutamine	2 + 2	+	132
Ammonium sulfate	1	—[1]	25
Ammonium sulfate	4	—	26
L-Glutamine	4	—	179
L-Glutamine	8	—	215

[1] Potassium nitrate was replaced by potassium chloride.

Table II. *Growth of Soybean Cell Cultures with Ammonium Salts as Sole Nitrogen Source*

The inoculum was 27 mg and the growing period was 5 days.

Compounds	Concn	Ammonium	Potassium Nitrate, 25 mM	Final Dry Weight	Final pH[1]
	mM	*mM*	*mM*	*mg*	
Ammonium sulfate (B5)	1	2	+	147	5.9
Ammonium sulfate	5	10	—[2]	29	3.5
Ammonium carbonate	3	6	—	34	4.5
L-Glutamine	5		—	90	4.6
Ammonium citrate	2	6	—	103	4.6
Ammonium citrate	4	12	—	115	4.4
Ammonium citrate	6	18	—	137	4.4
Ammonium succinate	5	10	—	157	5.3
Ammonium malate	5	10	—	178	5.1

[1] pH after the growing period.

[2] Potassium nitrate was replaced by 25 mM KCl.

5 μmoles of shikimate, 1.0 μmole of NADP, 50 μmoles of tris-(hydroxymethyl)methyl amino propane sulfonic acid buffer at pH 9.0, and enzyme in a total volume of 1.0 ml (4).

RESULTS

As shown by the data (Table I), some of the nitrogen sources supported cell growth better than others, but none was superior to the standard B5 medium. Nitrate alone or ammonium alone did not support growth. Some growth occurred on L-glutamine.

Table II shows the yields obtained after growing the cells in various ammonium salts. The cells did not grow on ammonium carbonate or sulfate alone. Ammonium citrate or malate supported growth approaching that obtained in the B5 medium. The concentrations of ammonium used in the experiments recorded in Table III were relatively low in comparison with the total nitrogen in the B5 medium, but the results show that ammonium salts of malate, citrate, succinate, or fumarate can be used. The data also suggest that malate does not enhance growth of the cells in a nitrate medium in the absence of ammonium.

Table IV shows the cell yields obtained on different ammonium compounds at higher concentrations of nitrogen. Equimolar amounts of ammonium to nitrate or glutamate at 10 mM were inferior to the B5 medium and indicate that ammonium at higher concentrations used without the Krebs cycle acids was inhibitory.

Table III. *Effect of Carboxylic Acids on Growth of Soybean Cell Cultures with Ammonium as the Sole Nitrogen Source*

The inoculum was 41 mg.

Carboxylic Acid	Concn	KNO_3	$(NH_4)_2SO_4$	Dry Weight	Final
	mM	*mM*	*mM*	*mg*	pH
None	...	25	...	73	6.3
None (B5)	...	25	1.0	251	6.4
Malate	5	25	0	84	7.3
Malate	5	...	5	191	5.1
Malate	5	...	10	129	4.2
Malate	10	...	10	178	4.6
Succinate	5	...	5	100	3.8
Citrate	5	...	5	206	5.1
Fumarate	5	...	5	156	4.9
Pyruvate	5	...	5	93	3.9

Table IV. *Growth of Soybean Cells on Different Ammonium Compounds*

The inoculum was 31 mg.

Compound	Concn of Nitrogen	Final Dry Weight	Final
	mM	*mg*	pH
K-nitrate + $(NH_4)_2SO_4$ (B5)	25 2	220	6.7
NH_4NO_3	20	137	4.4
Ammonium citrate	20	309	4.9
Ammonium malate	10	259	4.3
Ammonium shikimate	15	92	3.7
Ammonium glutamate	20	118	4.4
L-Glutamine	20	219	4.5
Ammonium tartrate	20	45	4.1

Table V. *Effect of Ammonium Sources on Growth and on Nitrogen-assimilating Enzymes in Soybean Cells Grown in Suspension Culture*

The medium contained no nitrate. Potassium was added as potassium chloride. Inoculum was 49 mg, and the growing period was 6 days for the first three samples and 9 days for the last four.

Compound	Nitrogen	Final Dry Weight	Enzyme Activity[1]			
			Gln-S	Glu-DH	iC-DH / NADP	Shik-DH
	mM	*mg*	*munits/mg protein*			
Ammonium citrate	20	400	620	5.50	7.38	2.81
Ammonium malate	20	380	780	7.88	8.00	2.35
Ammonium citrate Ammonium malate Ammonium shikimate	20	345	520	4.87	3.41	0.62
Ammonium shikimate	20	165	740	5.64	3.23	1.05
Ammonium tartrate	10	130	690	5.20	3.70	1.74
Ammonium sulfate	10	50	100	2.11	0.64	...[2]
Ammonium acetate	20	45	220	0.78	0.31	0
B5	27	224	1640	3.85	11.31	...

[1] Gln-S: Glutamine synthetase (EC 6.3.1.2); Glu-DH: glutamate dehydrogenase (EC 1.4.1.2); iC-DH: isocitrate dehydrogenase (EC 1.1.1.42); shik-DH: shikimate dehydrogenase (EC 1.1.1.25).

[2] Not determined.

The specific activities of glutamine synthetase, glutamate dehydrogenase, isocitrate dehydrogenase, and shikimate dehydrogenase were determined on cells grown on different ammonium salts (Table V). The data obtained with cells grown in the B5 medium are included for comparison. The specific activities of glutamine synthetase and isocitrate dehydrogenase were lower in cells grown on the ammonium salts alone than in cells grown in the B5 medium. The glutamate dehydrogenase concentrations were higher. Growth in the sulfate or acetate media was poor, and the specific activities of all enzymes were low. Similarly, shikimate and tartrate did not support growth, although the enzyme concentrations were as high as in cells grown in citrate or malate.

In addition to the soybean culture, we have cultured cells of flax and wheat in a citrate-ammonium medium through several subcultures. The medium was the same as the B5, but potassium nitrate and ammonium sulfate were replaced by 20 mM potassium chloride, 10 mM citric acid, and 20 mM ammonium hydroxide. The final pH of the medium was 5.5.

DISCUSSION

The soybean cells grew on ammonium salts as the sole nitrogen source provided citrate, malate, fumarate, or succinate was also present. The lack of growth on certain ammonium salts may be attributed to difficulties in absorption or to inhibition of metabolic reactions directly or indirectly associated with nitrogen assimilation. The ambient pH could be decisive for ammonium absorption (11), but there was no apparent correlation between pH and growth of the soybean cells in the various ammonium salts. Meister (10) has suggested that ammonium is absorbed as ammonia (NH_3) by microbial cells, and Croft (1) arrived at the same conclusion for the uptake of ammonium by chloroplasts. Croft showed that ammonium absorption was not affected by variation in pH. One function of the Krebs cycle acids in the growth of plant cells may be to facilitate ammonium uptake by providing a suitable electron flow system (1).

Ammonium in high concentrations disturbs the Krebs cycle in liver mitochondria (9). The conversion of isocitrate to α-ketoglutarate appeared to be particularly sensitive to ammonium. The ammonium salts also activated a mitochondrial reduced pyridine nucleotide pyrophosphatase which cleaves especially NADH (9). The net result would be a decrease in the levels of reduced pyridine nucleotides and α-ketoglutarate, thus limiting the rate of glutamate formation. Ammonium might affect similar enzyme systems in plant cells. The isocitrate dehydrogenase activity in the soybean cells grown on ammonium salts was low compared with that in cells grown on nitrate as the major nitrogen source. The activity of glutamate dehydrogenase was higher, suggesting that this enzyme was not a limiting factor. The activity of glutamine synthetase was lower in the ammonium-grown cells, but there was no indication that this enzyme restricted growth, since the activity in the ammonium citrate-grown cells was the same as that in the ammonium tartrate-grown cells. The present results support the view that in these cells the Krebs cycle acids facilitate ammonium utilization by stimulating ammonium-sensitive reactions directly involving the Krebs cycle acids. The formation of glutamate and glutamine possibly was not directly affected by ammonium.

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