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STUDIES ON EXCISED ROOTS OF *PHASEOLUS MUNGO* CULTIVATED *IN VITRO* AND *IN VIVO*

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Abstract—Studies were made of changes in chemical and enzyme composition of roots of *Phaseolus mungo* L. grown *in vitro* and *in vivo* with the progress of cultivation. The parameters measured were main axis length, dry weight, total and free sugar, amino acid nitrogen, RNA, DNA, free amino acids, and some of the enzymes of glycolytic and TCA cycle. The values for main axis length, number of laterals, total lateral length, dry weight, DNA, and glutamine were found to increase and those for amino acid nitrogen, RNA, alanine, aspartic acid and glutamic acid were found to decrease during a 7-day period of cultivation. The enzyme activities when expressed in terms of tissue nitrogen are generally found to show a decrease during cultivation, but the activities in the case of fumarate hydratase as well as most of the dehydrogenases are restored to original levels. Similar decreases are also found during cultivation of the roots *in vivo*. The differences in activity during *in vivo* and *in vitro* cultivations are generally in the same direction, except in the case of fumarate hydratase.

INTRODUCTION

STUDIES carried out on the metabolic status of cultivated roots[1–4] show that glucose and sucrose may be utilized for respiration and cellular synthesis by roots cultivated *in vitro*. These studies suggest that the root is a distinct metabolic entity. If the metabolic activity of roots cultivated *in vitro* is similar to that of roots grown *in vivo* the validity of the tissue culture technique for studies on simultaneous morphological and metabolic studies would be established. Also a systematic investigation of enzymes of carbohydrate metabolism and changes in the same during growth in excised roots cultivated *in vitro* would materially add to our comprehension of root metabolism.

In the present studies, optimum conditions were worked out for the cultivation of the roots of *Phaseolus mungo in vitro* and data obtained on the chemical and enzyme composition of the cultures at different stages of cultivation. Comparative data were obtained on roots grown *in vivo*.

RESULTS AND DISCUSSION

Changes with the progress of cultivation in main axis extension, lateral initiation, lateral extension, dry weight and nitrogen content of the root are shown in Table 1, from which it can be seen that lateral initiation is evident on the third day whereafter it increases steadily up to the seventh day. A similar steady increase in total lateral length and length of main axis is also evident. This is associated, as might be expected, with an increase in dry weight and total nitrogen content. The cultivation was therefore carried out for 7 days in subsequent experiments aimed at identifying the optimum conditions for cultivation. Maximum growth

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[3] W. P. POSTMA, *Proc. Acad. Sci. Amsterdam* **42**, 181 (1939).
[4] A. ULRICH, *Am. J. Botany* **28**, 526 (1941).

was obtained with a pH of 5·5 in a medium containing 2% sucrose and 100 mg/l $(NH_4)_2SO_4$. Further experiments were therefore carried out under these conditions. The analyses were carried out at zero time as well as on the second, fifth and seventh days (Table 2). The

TABLE 1. EFFECT OF PERIOD OF CULTIVATION ON GROWTH OF EXCISED ROOTS OF *Phaseolus mungo*

Period of cultivation (days)	Number of cultures	Mean growth values per root*			Average dry weight (mg)†	Average nitrogen content of five roots (mg)†
		Main-axis length (mm)	Number of laterals	Total lateral length (mm)		
0	10	10±0·2	0	0	2·5	0·12
1	10	20±1·6	0	0	5·8	0·21
2	10	40±0·7	0	0	7·1	0·23
3	10	61±0·8	1±0·2	5±0·6	9·0	0·33
4	10	86±1·5	4±0·3	15±1·2	11·4	0·45
5	10	108±1·6	9±0·6	40±2·0	16·2	0·66
6	10	117±1·6	13±0·4	57±3·6	21·2	0·91
7	10	127±1·7	14±0·4	76±4·2	28·2	0·97

* The values are given with the standard errors.
† Based on two batches consisting of five cultures each.

TABLE 2. CHEMICAL COMPOSITION OF ROOT CULTURE AT DIFFERENT STAGES OF CULTIVATION *in vitro*

	Days of cultivation			
	0	2	5	7
* Wet weight (mg)	90	152	381	480
* Dry weight (mg)	5·0	14·2	32·4	44·5
Moisture %	95·5	90·7	91·5	91·0
Value per 100 mg *dry weight*				
Nitrogen (mg)	4·80	3·22	4·25	4·34
Amino acid nitrogen (μg)	766	535	518	571
Free sugar (mg)	32·2	19·2	14·4	36·5
Total sugar (mg)	43·0	45·5	30·8	38·3
RNA (mg)	13·2	6·9	6·7	8·6
DNA (μg)	140	298	178	253
Alanine (μg)	292	246	259	166
Aspartic acid (μg)	136	99	35	24
Glutamine (μg)	85	56	293	186
Glutamic acid (μg)	128	105	138	93

* Values expressed for ten roots.

seventh-day values for nitrogen, free and total sugar, are found to be comparable to zero time values when expressed on dry weight basis, whereas the values for amino acid nitrogen, RNA, aspartic acid, alanine and glutamic acid show a decrease and that for DNA and glutamine show an increase. The changes are generally in the same direction during *in vitro* and *in vivo* cultivation except in the case of total sugar (Table 3).

TABLE 3. GROWTH AND CHEMICAL COMPOSITION OF ROOTS OF *Phaseolus mungo* CULTIVATED *in vitro* AND *in vivo*

	0 day	7th day	
		In vitro	*In vivo*
Main axis length (mm)	10±0·2	127±1·7	68±2·2
Number of laterals	0	14±0·4	6±1
Total lateral length (mm)	0	76±4·2	32±2
* Dry weight (mg)	5·0	44·5	27·4
Expressed per 100 mg *dry weight*			
Nitrogen content (mg)	4·80	4·34	5·12
Free sugar (mg)	32·2	36·5	40·7
Total sugar (mg)	43·0	38·3	70·3
RNA (mg)	13·2	8·6	5·0
DNA (μg)	140	253	194

* Values expressed for ten roots.

The activities of enzymes of carbohydrate metabolism during the progress of cultivation *in vitro* and *in vivo* are shown in Tables 4 and 5. In general, the activities of all the enzymes decrease on the second day suggesting perhaps the requirement of an adaptation period for cultivation *in vitro*. The enzyme activities are found to show a decrease when considered in terms of tissue nitrogen, but the activities in the case of fumarate hydratase as well as most of the dehydrogenases are restored to original levels. This does not happen in the case of the other enzymes. It must be pointed out that some decrease is also found during cultivation *in vivo*. The differences in activity during *in vivo* and *in vitro* cultivations are generally in the same direction except in the case of fumarate hydratase.

TABLE 4. TOTAL ACTIVITIES OF CERTAIN ENZYMES IN ROOT CULTURES OF *Phaseolus mungo* AT DIFFERENT DAYS OF CULTIVATION

Enzyme	Days of cultivation			
	0	2	5	7
Phosphoglucomutase	32·6	5·7	2·6	2·3
β-Fructofuranosidase	16·2	3·2	1·4	1·1
Hexokinase	2·9	1·3	0·6	0·5
Glucose-6-phosphate dehydrogenase	3·7	1·9	3·3	5·1
Lactate dehydrogenase	2·5	1·4	1·5	4·2
Pyruvate oxidase	1·5	0·3	0·4	0·5
Aconitate hydratase	5·5	1·8	1·5	3·6
Isocitrate dehydrogenase	5·5	2·6	4·3	3·6
Fumarate hydratase	5·9	2·6	4·3	8·4
2-Oxoglutarate oxidase	3·3	1·4	0·9	1·0
Glutamate dehydrogenase	5·9	2·7	3·1	6·7
Glutamine synthetase	1·1	0·5	0·4	0·4
γ-Glutamyltransferase	13·6	5·2	3·2	5·9
Alanine aminotransferase	14·2	3·8	1·8	2·2
Aspartate aminotransferase	17·8	4·9	3·4	2·8

Values expressed as units/mg N_2 of root tissue.

TABLE 5. TOTAL ACTIVITIES OF CERTAIN ENZYMES IN ROOTS OF *Phaseolus mungo* CULTIVATED *in vitro* AND *in vivo*

Enzyme	7th day	
	In vitro	*In vivo*
β-Fructofuranosidase	1·1	1·0
Hexokinase	0·5	Not determined
Phosphoglucomutase	2·3	3·3
Glucose 6-PO_4 dehydrogenase	5·1	6·7
Lactate dehydrogenase	4·2	8·0
Pyruvate oxidase	0·5	Not determined
Aconitate hydratase	3·6	6·3
Isocitrate dehydrogenase	8·4	14·0
Fumarate hydratase	8·4	4·1
2-Oxoglutarate oxidase	1·0	Not determined
Glutamate dehydrogenase	6·7	11·8
Glutamate synthetase	0·4	1·0
γ-Glutamyl transferase	5·9	10·8
Alanine aminotransferase	2·2	5·3
Aspartate aminotransferase	2·8	5·9

Values expressed as units/mg N_2 of root tissue.

In conclusion, the results show the metabolic capacity of the root tissue to grow and synthesize most of its cellular constituents and the metabolic similarity of root tissue cultivated *in vitro* to that grown *in vivo*.

EXPERIMENTAL

Seeds

Seeds of *Phaseolus mungo* (Var. urdi N.P.4) were obtained from the Botanical Sub-station of the Agricultural Research Institute, Pusa, Bihar, India.

Germination of Seeds

All the operations to be described below were carried out under aseptic conditions in a sterile room maintained at a temperature of 26 ± 1°. Glass-distilled water was used for all the studies. Seeds were surface sterilized by immersion in 50 ml of 0·1% aq. $HgCl_2$ for 5 min, and in 50 ml of a fungicide solution containing 50% v/v alcohol, 20% v/v thymol and 5·0% v/v glycerol for 2 min. The fungicide was removed by successive washings with water and 100 seeds were sprayed over a layer of Whatman No. 1 paper in a 9 cm Petri dish containing 4·5 ml of water. After 20 hr, 1·5–2·0 ml more of water was added to the Petri dish. The seeds were germinated at 26 ± 1° in the dark for 40 hr.

Medium

White's medium[5] with the following modifications was used for the cultivation of *P. mungo* roots: (*a*) Molybdic acid and $CuSO_4$, $5H_2O$ were added at concentrations of 0·0017 mg and 0·013 mg per l. respectively as recommended by Boll and Street.[6] (*b*) $(NH_4)_2SO_4$ was required at the concentration of 100 mg per l. to achieve growth. (*c*) 0·6% agar was used in the medium. Double glass-distilled water was used for preparing the medium. The pH of the agar medium was adjusted to 5·5 at 40–50°. 50 ml of the medium were distributed to each test tube (2·5 × 20 cm) and plugged with cotton wrapped in gauze cloth. The medium was sterilized by autoclaving at 15 lb/in² for 10 min.

Inoculation and Cultivation of Roots in vitro

Excisions of root tips and subsequent inoculations were carried out in an aseptic cabinet using a sterilized razor blade and a stainless-steel spatula. Root tips of about 10 mm length were excised from the seedlings

[5] P. R. WHITE, *A Handbook of Plant Tissue Culture*, Jaques Cattell Press, Lancaster, Pennsylvania.
[6] W. C. BOLL and H. E. STREET, *New Phytol.* **50**, 51 (1951).

TABLE 6. METHODS USED FOR CHEMICAL ANALYSIS AND ENZYME ASSAYS

Parameter measured	Method used
(*a*) *Chemical analysis*	
Total and free sugar	Nelson[7] and Somogyi[8]
Amino acid nitrogen	Russel[9]
RNA	Orcinol method[10]
DNA	Diphenylamine method[10]
Free amino acids	Hakkinen and Kulonen[11]
(*b*) *Enzyme assays**	
α-Glucanphosphorylase	Whelan[12]
Phosphoglucomutase	Najjar[13]
β-Fructofuranosidase	Straus[14]
Hexokinase	Sharma *et al.*[15]
Lactate dehydrogenase	Kornberg[16]
Pyruvate oxidase	Varma [17]
2-Oxoglutarate oxidase	Varma [17]
Aconitate hydratase	Racker[18]
Fumarate hydratase	Racker[18]
Isocitrate dehydrogenase	Ramakrishnan and Martin[19]
Succinate dehydrogenase	Srinivasamurthy and Swaminathan[20]
Malate dehydrogenase	Srinivasamurthy and Swaminathan[20]
Glutamate dehydrogenase	Bulen[21]
Glutamine synthetase	Elliot[22]
Glutamyl transferase	Waelsch[23]
Alanine aminotransferase	Shah and Ramakrishnan[24]
Aspartate aminotransferase	Shah and Ramakrishnan[24]
Glucose-6-phosphate dehydrogenase	Chakravorty and Burma[25]

* In all the cases except hexokinase, dehydrogenases, pyruvate oxidase and 2-oxoglutarate oxidase enzyme unit is defined as the amount required for the disappearance of 1 μmole of the substrate or formation of 1 μmole of product in 60′ under the conditions of assay. In case of hexokinase and dehydrogenases it is defined as the amount required for the reduction of 1 μmole of NAD, NADP or TTC or oxidation of 1 μmole $NADH_2$ or $NADPH_2$ in 60′ under the conditions of assay. In the case of pyruvate oxidase and 2-oxoglutarate oxidase it is defined as the amount required for the utilization of 1 μmole of the O_2 in 60′ under the conditions of the assay.

[7] N. NELSON, *J. Biol. Chem.* **153**, 375 (1944).
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[25] M. CHAKRAVORTY and D. P. BURMA, *Biochem. J.* **73**, 48 (1959).

and inoculated individually into the culture medium to initiate the root cultures. The cultures were incubated at 26 ± 1° in the dark for 7 days. The cultures were primary cultures and attempts at serial subculturing were not successful.

Cultivation of Roots in vivo

Small flower-pots containing garden soil were sterilized at 15 lb/in² for 15 min. These pots were taken to the culture room (maintained at 26 ± 1° with adequate lighting) and 200 surface sterilized seeds as described earlier were sown in each pot and allowed to germinate in sterile, cold, distilled water and the roots cut and used for chemical and enzyme analyses.

Measurement of Growth

The growth of the cultures was judged in terms of the length of the main axis, number of laterals, total length of laterals, dry weight and nitrogen content. The length of the main axis and the laterals was measured using a transparent scale. The cultures were taken out and washed free of agar in water and oven dried at 60° for 24 hr. Nitrogen content was estimated by the microkjeldahl method. Pooled samples of five cultures each were taken for the determination of dry weight and nitrogen content. Values per root were calculated using the mean of two samples.

Preparation of Root Extract for the Estimation of Free Amino Acids

The root extract for the estimation of free amino acids was prepared by the method described by Pillai and Srinivasan.[26]

Preparation of Root Extract for Enzyme Assay

All the operations described below were carried out at 0–4°. Fresh roots were washed with buffer, pressed gently between filter papers to remove as much water as possible and ground in a chilled mortar with three times the volume of 0·02 M potassium phosphate buffer, pH 7·0, for 15 min. The crude homogenate was first filtered through cheese cloth and centrifuged at 3860 × *g* and 0° for 15 min. The supernatant was used for the estimation of the activities of hexokinase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, aconitate hydratase, isocitrate dehydrogenase and glutamate dehydrogenase. In the case of aspartate aminotransferase and alanine aminotransferase, the supernatant was first dialysed for 3½ hr against 0·02 M potassium phosphate buffer, pH 7·0.

For the estimation of glutamine synthetase and glutamyl transferase the enzyme extract was prepared using 0·02 M sodium bicarbonate buffer, pH 8·0, as a grinding medium in place of potassium phosphate buffer. For the estimation of the activities of malate dehydrogenase and succinate dehydrogenase crude phosphate buffer homogenate was used without centrifugation. For the estimation of the activities of β-glucanphosphorylase and phosphoglucomutase enzyme extract was prepared using water as a grinding medium. In the case of β-fructofuranosidase crude 10% KCl (0·9%) extract was used without centrifugation.

The methods used for chemical analyses and enzyme estimation are indicated in Table 6.

[26] N. C. PILLAI and K. S. SRINIVASAN, *J. Gen. Microbiol.* **14**, 248 (1956).