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PRIMARY METABOLITES OF *PHASEOLUS VULGARIS* NODULES

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; host cell; bacteroid; carbohydrate; amino acids; organic acids.

Abstract—More ethanol soluble material (carbohydrate and amino nitrogen) was found in both host cell and bacteroid components of *Phaseolus vulgaris* nodules from plants grown at 28 W/m^2 than from plants grown at 7 W/m^2. The range of compounds identified was similar at the two irradiances. On feeding $^{14}CO_2$ to the plant tops at either irradiance the labelling patterns of carbohydrates and organic acids in the nodule host cells and bacteroids suggested that any or all of the following substances could be donated by the host to the bacteroids for general metabolism: sucrose, fructose, glucose, an unidentified carbohydrate, malic acid and an organic acid co-chromatographing with 6-phosphogluconate. Distribution and labelling patterns of nodule amino compounds were consistent with the hypothesis that ammonia is the primary product of nitrogen fixation within bacteroids, and that this ammonia is transported to host cells for assimilation, initially into glutamine and glutamate.

INTRODUCTION

Little is known of the levels of individual sugars and/or organic acid levels in root nodules, or how these relate to nodule activity [1–5]. As part of a general study into the effects of irradiance on growth and nitrogen fixation in *Phaseolus vulgaris* it was found that nitrogen fixing activity per unit of nodule wt was not greatly affected by irradiance. However nodule growth and development was dependent on irradiance [6]. Since light was expected to affect photosynthate supplied, levels of both carbohydrate and amino nitrogen in the two symbiotic components of nodules, host cells and bacteroids, were examined.

RESULTS

Total ethanol soluble carbohydrate and amino nitrogen levels were higher in host cells than in bacteroids, and in both nodule components levels of these metabolites were greater at 28 W/m^2 than at 7 W/m^2. Amounts of carbohydrates per g ethanol soluble material were unaffected by irradiance, and the levels in bacteroids were only slightly lower than those in host cells. Levels of amino nitrogen per g ethanol soluble material were lower in bacteroids than in host cells, but again no irradiance effect was noted (Table 1).

The major amino compounds in host cells were ammonia, glutamic acid and citrulline, whereas in bacteroids the ammonia content far surpassed that of other amino compounds, the proportion of glutamine in relation to other amino acids was higher, and citrulline was absent (Table 2). In terms of μmol of individual amino compounds, levels were generally higher at 28 W/m^2 than at 7 W/m^2. However, in terms of μmol per g of ethanol soluble material (assuming equal losses in all treatments) a different pattern was seen (Table 3). Host cell citrulline, lysine and ammonia and bacteroid glutamine, ammonia, threonine and serine were higher at 7 W/m^2 than at 28 W/m^2. Glutamic acid, γ-amino-

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Table 1. Total ethanol soluble carbohydrate and amino nitrogen levels in host cells and bacteroids of nodules of *P. vulgaris* plants grown at 7 or 28 W/m^2

Age (days)	7 W/m^2								28 W/m^2							
	Carbohydrate				Amino nitrogen				Carbohydrate				Amino nitrogen			
	mg/plant		mg/g*		mg/plant		mg/g*		mg/plant		mg/g*		mg/plant		mg/g*	
	B	H	B	H	B	H	B	H	B	H	B	H	B	H	B	H
32	0.02	0.68	325	668	0.003	0.08	45.7	78.6								
33									0.04	1.62	102	470	0.032	0.23	91.5	65.5
39		1.02		283		0.27		74.8	0.14	1.97	239	261	0.034	0.41	59.0	53.8
40	0.20		223		0.033		37.6									
48	0.24	1.67	417	458	0.028	0.29	48.1	79.2								
50	0.26	1.95	383	413	0.034	0.30	50.8	63.7	0.51	4.79	304	451	0.048	0.81	28.5	77.6
60									0.57		336		0.067		39.7	
63	0.12		284		0.014		33.3									
74		1.79		389		0.39		85.7		3.31		517		0.39		61.5

* mg/g = mg metabolite/g EtOH soluble material. B = bacteroid fraction. H = host cell fraction.

Table 2. Amino acid composition of host cells and bacteroids of pink nodules of plants grown at 7 and 28 W/m²

$$\text{Values} = \frac{\text{mg of amino acid per g EtOH soluble material}}{\text{total mg amino acids per g EtOH soluble material}} \times 100$$

	Host cells		Bacteroids	
	7W/m²	28 W/m²	7W/m²	28 W/m²
Aspartate	10.1	8.8	0.7	2.8
Threonine	3.4	3.4	0.2	T
Serine	4.3	3.7	0.5	T
Glutamate	17.1	29.7	3.8	2.8
Glutamine	6.4	4.5	5.0	2.8
Citrulline	17.7	8.8	—	—
Glycine	T	2.1	0.9	1.8
Alanine	1.8	5.3	T	2.1
Valine	2.7	2.7	T	4.2
Methionine	0.6	0.3	—	—
Isoleucine	4.9	5.8	2.9	4.6
Leucine	5.2	7.4	3.4	7.1
Tyrosine	1.2	1.1	*	*
Phenylalanine	0.9	1.3	*	1.4
γ-Aminobutyrate	2.1	4.2	1.1	1.4
Putrescine	T	T	T	—
Histidine	0.9	0.8	—	—
Lysine	6.4	2.7	—	—
Ammonia	14.3	7.4	81.6	68.7

T = trace amounts, * = found in later experiments. The following were also present in both fractions in more than trace amounts but were not quantified due to incomplete separation: asparagine, cystine, ornithine, arginine. One unidentified amino acid ran before aspartic acid and was found in large amounts in the host cells and to a lesser degree in bacteroids.

butyric acid, leucine, alanine and glycine of host cells, and leucine, glycine, alanine, valine and aspartic acid of bacteroid cells were substantially greater at 28 W/m² than at 7 W/m². Others were similar at both irradiances.

Of the label in the nodules after the cold chase period 92–95% was located in the host cell material and 5–8% in the bacteroids, irradiance having no effect on this distribution. In both host cells and bacteroids the bulk (60–85%) of the label went into the ethanol soluble neutral fraction. In the bacteroids the proportion of label in the basic fraction (11% at 28 W/m²) was greater than that in the acidic fraction (6% at 28 W/m²), whereas in host cells the situation was reversed (the basic fraction having 14% of the label and the acidic 22% at 28 W/m²).

Distribution of label between individual metabolites is given in Table 4. The major labelled compounds of each fraction were: sucrose, malate and glutamate (or glutamine) in host cells, and unidentified carbohydrate 'A', the organic acid which co-chromatographed with 6-phosphogluconate (being the only organic acid found in the bacteroid extracts), and glutamate (or glutamine) in bacteroid cells. Also shown in Table 4 are the effects of the two irradiances on the distribution patterns.

DISCUSSION

Higher irradiance levels give higher carbohydrate levels in nodules of some species [7–10], and the results obtained here for *P. vulgaris* showed that although total ethanol soluble carbohydrate levels were greater at 28 W/m² than at 7 W/m² this resulted from the greater amounts of nodule material at the higher irradiance level.

The same carbodydrates were present in host cells and bacteroids at both irradiances. The effects of irradiance on labelling patterns were variable, but generally greater values at 28 W/m² were due to larger amounts of material at this irradiance. (Table 5).

The major sugar in host cells of pink nodules was sucrose, as found in *Glycine max* [5, 7] but not in *Vicia faba* nodules [3]. In ^{14}C-feeding experiments this sucrose was heavily labelled (60% of the total host cell neutral fraction label). This is consistent with the correlation found by Streeter and Bosler [5] between sucrose content and rates of acetylene reduction. However, this does not necessarily indicate that host cells donate sucrose as such to bacteroids, especially as host cells of other legumes contain high levels of invertase activity [11, 12]. Alternatively, other highly labelled carbohydrates (glucose, fructose, 'A') may be transferred from host cell to bacteroids.

Organic acids provide another possible source of metabolites for bacteroids. Stimulation of nitrogen fixation *in vitro* by organic acids has been demonstrated

Table 3. Comparison of total μmol and μmol/g EtOH soluble material for the major amino compounds in pink nodule fractions from plants grown at 7 and 28 W/m²

	Total μmol of amino compounds				μmol of amino compound/g EtOH soluble material			
	Host cell		Bacteroid		Host cell		Bacteroid	
	7 W/m²	28 W/m²	7 W/m²	28 W/m²	7 W/m²	28 W/m²	7 W/m²	28 W/m²
Ammonia	23.5	27.0	25.5	35.2	276	164	2128	1134
Glutamate	6.3	18.5	0.7	2.2	74	112	58	71
Glutamine	1.2	1.9	0.2	0.2	15	12	15	5
Citrulline	2.8	3.1	0	0	34	19	0	0
Lysine	1.2	1.1	0	0	14	7	0	0
Threonine	0.8	1.9	0.1	0	9	11	1	0
Serine	1.2	2.2	0.2	0	13	13	2	0
γab*	0.5	2.6	0.1	0.2	7	16	5	4
Leucine	1.1	3.5	0.1	0.5	13	21	11	15
Alanine	0.5	3.8	0	0.2	6	23	0	6
Glycine	0.2	1.9	0.1	0.2	1	11	5	8
Valine	0.6	1.4	0	0.3	3	8	0	10
Aspartate	2.1	4.1	0.1	0.2	25	25	3	6

* γab = γ-aminobutyric acid.

Table 4. Patterns of labelling of carbohydrates, amino acids and organic acids from pink nodules of plants grown and fed $^{14}CO_2$ at 7 or 28 W/m^2. Values are the percentage of the total amount of ^{14}C in the fraction located in each compound

	Host cells		Bacteroids	
	7 W/m^2	28 W/m^2	7 W/m^2	28 W/m^2
Carbohydrates				
Sucrose	61.5	76.0	17.0	16.0
Glucose	22.0	11.3	13.0	18.0
Fructose	10.0	5.0	14.0	9.0
Sugar alcohol	0.6	0.7	2.0	3.0
Arabinose	0.9	0.8	15.0	5.0
'A'	4.3	4.2	37.0	45.0
'B'	0.6	0.7	2.0	2.0
'C'		1.2		
Organic acids				
Citrate	7.5	9.5		
Malate	53.6	33.2		
Malonate	16.9	19.7		
Uroconic	5.3	7.3		
$R_f = 0$	2.9	4.3		
$R_f = 5$	3.0	20.1	100	100
$R_f = 77$–82	10.8	5.9		
Amino acids				
Glutamic acid	49.2	44.5	27.3	35.6
Glutamine	5.4	3.0	30.6	14.7
Asparagine	10.4	6.4		6.4
Threonine	5.3	5.1		
Alanine	4.8	7.6		
Phenylalanine	4.6	5.3		
Valine	4.1	6.5		
Aspartic acid	2.2	1.9		
Isoleucine/leucine	1.5	0.9	23.1	7.2
Cystine	1.3	1.6	12.4	3.7
γ-Aminobutyrate(?)	11.1	17.3	6.6	17.6
'X'				14.7

Carbohydrate: (1) the sugar alcohol was not further identified. (2) 'B' had R_g = 154–160 in *iso*-PrOH–H_2O (4:1). (3) 'C' had R_g = 138–146 in *iso*-PrOH–H_2O (4:1). (4) 'A' had R_g = 64–70 in *iso*-PrOH–H_2O (4:1), and 53 in *n*-PrOH–EtOAc–H_2O (7:1:2). It was possibly an aldose, disaccharide, non-reducing sugar. Organic acid: R_f values given are those in water satd *n*-PrOH–HCO_2H–eucalyptol (5:2:5). The acid running at $R_f = 5$ co-chromatographed with 6-phospho-gluconate, and that of R_f = 77–82 was possibly itaconic acid. Amino acid: The spot tentatively given the identity γ-aminobutyric acid ran in the expected position of that compound but was ninhydrin negative. 'X' was a streak running close to the solvent front in *n*-BuOH–HOAc–H_2O (12:3:5).

[13, 14], and Lawrie and Wheeler [4] have reported fixation of carbon dioxide to organic acids (e.g. malate) by detached *V. faba* nodules in the dark, with concurrent amino acid synthesis. In the present work host cell malate was highly labelled, although not produced by fixation in the nodules as the roots were not in contact with $^{14}CO_2$. Malate was not detected in the bacteroids, suggesting rapid metabolism if this should be a carbon donor. The only organic acid detected in the bacteroids (co-chromatographing with 6-phosphogluconate) was also highly labelled in host cells of 28 W/m^2 plants: this could be the species donated by host to bacteroids.

As with carbohydrate, the free amino content per g ethanol soluble material was similar at both irradiances. It is generally accepted that ammonia synthesised in

Table 5. Levels of ^{14}C in the major carbohydrates and organic acids of host cells of pink nodules from plants grown and fed $^{14}CO_2$ at 7 or 28 W/m^2

	μCi/μCi in total fraction		μCi/μCi in total fraction/g EtOH soluble material	
	7 W/m^2	28 W/m^2	7 W/m^2	28 W/m^2
Sucrose	0.015	0.032	0.622	0.581
Glucose	0.005	0.005	0.207	0.091
'A'	0.001	0.002	0.046	0.033
Malate	0.071	0.043	4.48	1.22
$R_f = 0$	0.004	0.006	0.239	0.160
$R_f = 5$	0.004	0.026	0.252	0.736

bacteroids moves into the host cells where it is converted to glutamine and glutamic acid by the action of glutamine synthetase and glutamate synthase [13, 15, 16, 17]. The following data obtained in this work support this pathway.

1. Bacteroid ammonia formed a much greater proportion of free amino nitrogen than host cell ammonia. However, this must be regarded with caution as several investigators [18–20] have noted large pool sizes in comparison to the amounts of ^{15}N-ammonia produced in labelling experiments, and have postulated the existence of more than one ammonia pool in nodules.

2. During the course of this work it was found that bacteroids 'leaked' large amounts of amino compounds (7–8% of total ethanol soluble amino nitrogen in 3 hr, compared to 1% of total ethanol soluble carbohydrate in the same time) into both water and buffer (phosphate, pH 7, containing 1 mM magnesium sulphate and 0.3 M sucrose). Of the leaked amino compounds ammonia was the major component, and in smaller amounts were glutamate, leucine and isoleucine [6].

3. Glutamate plus glutamine levels (considered together due to variable breakdown of glutamine in hydrochloric acid) and their ^{14}C-labelling patterns stressed their importance in the formation of amino acids from ammonia. Their concentrations were generally greater in host cells than in bacteroids and the amount of label in host cell glutamate per g ethanol soluble material was 7 times that in bacteroid glutamate.

Ammonia levels per g ethanol soluble material were smaller at 28 W/m^2 than at 7 W/m^2 in both fractions. This might indicate more rapid turnover of ammonia at high irradiance. If so, acetylene reduction data as a measurement of the rate of nitrogen fixation must be critically reviewed as the possible limitation of ammonia utilisation is not reflected by the reduction of acetylene to ethylene [6].

Amino nitrogen is transported from legume nodules to the rest of the plant as asparagine [17, 21–23], which is thought to be formed from aspartic acid by the action of glutamine dependent asparagine synthetase [24]. In this work aspartic acid constituted a high proportion of host cell amino nitrogen although it was labelled to a lesser degree than glutamine and glutamic acid; host cell asparagine was highly labelled. This indicates a large aspartic acid pool size and rapid turnover of aspartic acid to asparagine

Another amino acid of interest was γ-aminobutyrate. This was found in much lower proportions in relation to other amino acids than found by Butler and Bathurst

[25] for *Trifolium spp.* As reported by Freney and Gibson [26] for bound γ-aminobutyric acid, levels were lower in bacteroids than in host cells, but the effect of irradiance on levels per g ethanol soluble material (i.e. levels at 28 W/m^2 greater than those at 7 W/m^2) were opposed to those found by the above authors. A ninhydrin negative compound running in the expected position of γ-aminobutyric acid in butanol–acetic acid–water was highly labelled. Butler and Bathurst [25] reported ninhydrin negative reactions of γ-aminobutyrate under phenolic conditions, and a high label in this compound would be expected if it is synthesised from glutamic acid, as reported by Virtanen and Miettinen [27] and Freney and Gibson [26].

Technical difficulties, such as the time required to harvest sufficient material, the small proportion of ^{14}C entering the bacteroid fraction, and the leakage of bacteroid metabolites during long centrifugations, prevented turnover studies with *P. vulgaris* nodules. Thus the pathways suggested are not proven: they are however consistent with other published work and give a framework for further experimentation.

EXPERIMENTAL

Plant material. Seeds of *Phaseolus vulgaris* (cv Glamis) were inoculated with a suspension of *Rhizobium phaseoli* and germinated in vermiculite. Vigorous seedlings were transplanted into sterile sand and grown at two irradiance levels (7 and 28 W/m^2) in growth cabinets [6]. Once the first leaves had expanded, plants were watered with nitrate-free nutrient soln. Plants were harvested at various ages, but always 10 hr after the beginning of the photoperiod. 18 plants per irradiance were used for host cell metabolite and 48 plants per irradiance for bacteroid metabolite determination. 7 and 28 W/m^2 plants were harvested on consecutive days. For $^{14}CO_2$ uptake experiments 40- to 48-day-old plants (at or near their peak of nitrogen fixing activity) were used. 12 plants per irradiance were used for host cell and 48 plants per irradiance for bacteroid metabolite determination, all plants being exposed to $^{14}CO_2$ 10 hr after the beginning of a photoperiod. 7 and 28 W/m^2 plants were harvested within 2 days of each other.

Separation of bacteroid and host cytoplasm fractions. Washed root systems were kept on ice and pink nodules quickly removed. The central region of the pink nodules was removed by cutting the nodules in half and gently crushing in H_2O. Homogenates were filtered through 4 layers of muslin and filtrates centrifuged at 3000 *g* for 15 min to give a host cell cytoplasm supernatant and a bacteroid pellet [6]. The fractions were separated and the bacteroids disrupted to release cell contents by use of a French press at 11 × 10^7 N/m^2.

Extraction of cell carbohydrates, amino acids and organic acids. The cell sap and lysed bacteroid fractions were extracted in 80% EtOH at 4° for 48 hr. EtOH soluble material was separated by centrifugation, dried by evapn at red. pres. (30–35°) and redissolved in a small vol. of 80% EtOH.

Separation of acid, basic and neutral substances from EtOH extracts. Ion exchange resins, Dowex 50X8-400 and Dowex 1X8-400 [1, 28, 29] were used. After application of samples non-adhering material was washed through the column with 80% EtOH, and adhering material was eluted with either 1.5 N HCl (Dowex 50) or 4 N HCOOH (Dowex 1), both followed by more 80% EtOH [6]. Separated fractions were dried by evapn at red. pres. (30–35°). The basic fraction was washed until neutral with 80% EtOH; however, breakdown of glutamine to glutamate still occurred. Samples were redissolved in 1 ml 80% EtOH.

Determination of EtOH soluble carbohydrate and amine compounds. Carbohydrate was determined by the anthrone assay using D-glucose standards [30], and amino acids by a modified ninhydrin method, with L-leucine standards [31].

Amino acid analysis. 0.1–0.4 ml aliquots of the basic fractions were put through a BIOCAL BC 100 amino acid analyser [6].

PC. A variety of systems were used for each group of metabolites [6]. In all cases Whatman No. 1 chromatography paper was used. Co-chromatography of eluted (in 80% EtOH) spots was used for identification.

^{14}C-labelling of nodule metabolites. Entire plant tops were fed $^{14}CO_2$ for 30 min in perspex boxes within the growth cabinets, the gas being liberated by addition of HCl to Na$H^{14}CO_3$ (58.9 mCi/mmol) in such quantities as to give 10 μCi/plant. At the end of the feed period $^{14}CO_2$ was removed from the box and a 2.5 hr cold chase period was given (to ensure adequate labelling of metabolites). Plants were harvested and metabolites extracted and processed as previously described. At intervals 10 or 100 μl aliquots were taken for scintillation counting in PPO–POPOP–toluene–triton (aq.) or PPO–POPOP–toluene (vacuum desiccated EtOH solns) cocktails. The activity of the chromatographically separated compounds was measured by cutting out the stained spots and counting in PPO–POPOP–toluene scintillant (autoradiographs were used to give accurate location of labelled material).

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