Phytochemistry, 1967, Vol. 6, pp. 321 to 328. Pergamon Press Ltd. Printed in England

HOST PHYSIOLOGY AS RELATED TO NODULATION OF SOYBEAN BY RHIZOBIA*

D. H. HUBBELL† and G. H. ELKAN

Department of Microbiology, Institute of Biological Sciences,
North Carolina State University at Raleigh

(*Received* 9 *August* 1966)

Abstract—Host physiological factors possibly related to nodulation of legumes by rhizobia were investigated. Comparison of biochemical patterns in inoculated and uninoculated root material from a normal, nodulating (NN) soybean, and a mutant, non-nodulating (nn) soybean revealed some distinct differences. During the days preceding visible nodulation, roots of uninoculated NN plants contained larger amounts of protein and reducing sugars and smaller amounts of free amino acids than did uninoculated nn roots. Amounts of protein, free amino acids and reducing sugars decreased more rapidly in inoculated NN and nn plants than in the corresponding uninoculated plants. In the case of free amino acids and reducing sugars this decrease occurred much earlier in inoculated nn plants. High carbohydrate/nitrogen ratios were found in plants which were susceptible to nodulation; low carbohydrate/nitrogen ratios were found in nodulation-resistant plants.

INTRODUCTION

RECENT investigations in several laboratories present a detailed description of invasion and nodulation of leguminous plant roots by rhizobia. However, the biochemical interactions of the host plant and the rhizobia which are required for nodulation are unknown. The presence and importance of such biochemical factors is evident from the unique specificity of different *Rhizobium* spp. for certain host plants.

Physiological studies of the nodulation process have become possible through the acquisition of (1) a normal, nodulating soybean strain, and (2) a near-isogenic mutant, non-nodulating soybean strain. The present investigation is an attempt to identify physiological properties which are required for or inhibitory to infection and nodulation of leguminous plants by rhizobia.

The single gene mutation in the nn soybean used in this study prevents nodulation by infective rhizobia, possibly by altering the physical or chemical properties of roots of the nn plants. Clark[1] and Elkan[2] found rhizobia on and in roots of this soybean. Hubbell[3] found no evidence of a physical barrier to nodulation as evidenced by normal root hair numbers and structure. However, Elkan[4] reported a nodulation-inhibiting factor in root exudate of nn plants and Hubbell[3] found an adverse effect of the exudate on formation of the 'swarmer' stage of the rhizobia reported to cause plant infection. Investigations were therefore directed toward detection of some alteration in the chemical composition of nn plant roots available for invasion by rhizobia. This was accomplished by comparison of patterns

* Contribution from Microbiology, North Carolina Agricultural Experiment Station, Raleigh, North Carolina. Published with the approval of the Director of Research as Paper No. 2233 of the Journal Series.

† Present Address: Department of Agronomy, Cornell University, Ithaca, New York.

[1] F. E. CLARK, *Can. J. Microbiol.* 3, 113 (1957).
[2] G. H. ELKAN, *Can. J. Microbiol.* 8, 79 (1962).
[3] D. H. HUBBELL, Unpublished M.S. Thesis, North Carolina State University, Raleigh (1962).
[4] G. H. ELKAN, *Can. J. Microbiol.* 7, 851 (1961).

of change in chemical composition in non-inoculated and inoculated roots of the normal and mutant soybeans.

RESULTS AND DISCUSSION

Since the two soybean strains used in this study do not differ phenotypically, it was expected that the homozygous recessive gene blocking nodulation expresses itself as a quantitative change in a metabolic pathway. Gross chemical composition of seed material was determined in order to detect possible differences in minerals or food reserves which might influence the course of the nodulation process.

No differences were found in results of analyses of NN and nn soybean seed for major minerals (Ca, Mg, P, K, S, N), protein, lipid and carbohydrate. Very similar amounts of all 17 amino acids determined were detected in hydrolysates of the two seed proteins. Thus differences in the chemical composition of roots of NN and nn plants cannot be traced to any obvious difference in the gross chemical composition of the seeds of these two strains of soybean.

Soybeans inoculated at planting show visible nodules within 9–10 days. The nn plant therefore must establish its barrier to nodulation at germination or very shortly thereafter. Comparison of analyses from non-inoculated root material of the two plant strains should reveal any gross biochemical differences. This could indicate the nature of the mutation blocking nodulation in the nn strain. Comparisons between inoculated and non-inoculated roots of both NN and nn plants should indicate whether or not there is any resistant reaction by the plants in response to rhizobial infection and whether or not the response is the same quantitatively and/or qualitatively for both plant strains.

TABLE 1. AMINO ACIDS IN DRIED ROOT MATERIAL OF INOCULATED AND UNINOCULATED NN AND nn SOYBEANS

Amino acid	μmoles/g			
	NN uninoculated	NN inoculated	nn uninoculated	nn inoculated
Lysine	66	68	80	76
Histidine	37	37	42	41
Ammonia	387	438	349	367
Arginine	46	48	46	43
Aspartic acid	367	377	275	341
Threonine	50	56	45	54
Serine	68	75	67	76
Glutamic acid	89	102	86	95
Proline	58	67	60	69
Glycine	79	88	74	82
Alanine	78	89	75	84
Valine	66	75	68	72
Methionine	12	14	11	12
Isoleucine	49	55	49	54
Leucine	73	82	74	81
Tyrosine	25	27	30	31
Phenylalanine	39	43	36	42
Total amino acids: % of sample	16·08	17·41	14·99	16·70

There were no qualitative differences in sugars in alcoholic root extracts from the four groups of plants. Since amino acids have been implicated in attenuation of virulence of rhizobia,[5] inhibition of nodulation[6] and induction of pleomorphic or bacteroid forms of rhizobia[7] these compounds were investigated. No qualitative differences in amino acid composition were detected in hydrolysates of dried roots of inoculated or uninoculated NN or nn plants. Quantitative analysis of these hydrolysates (Table 1) revealed that most amino acids were present in similar amounts in uninoculated NN and nn plant roots, although ammonia and aspartic acid were present in larger amounts in NN plant roots. These data indicate a similarity in amino acid metabolism but the analysis did not distinguish between free and protein-bound amino acids. Levels of amino acids in inoculated roots of both plant strains were comparably higher than levels found in the corresponding uninoculated roots. However, ammonia showed the highest increase in inoculated NN plants. It is possible that plant infection stimulates mobilization of amino acids from the cotyledons in both plant strains. It is also possible, however, that the increase in amino acids in inoculated NN plants

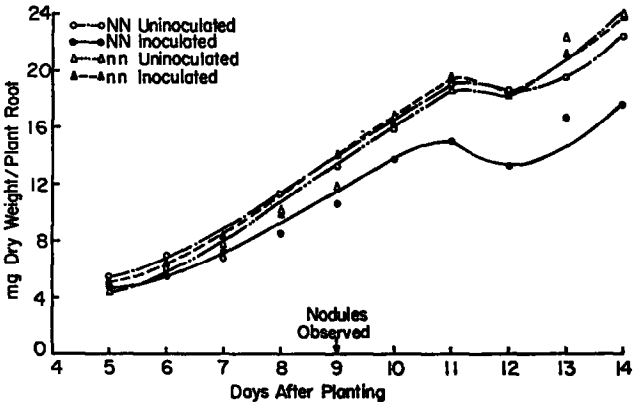

FIG. 1. TOTAL DRY WEIGHT (MG/PLANT ROOT) PER ROOT OF FOUR SOYBEAN TREATMENTS HARVESTED FOR SUBSEQUENT ASSAYS. EACH POINT REPRESENTS THE AVERAGE OF TEN PLANTS.

is due primarily to an increase in available nitrogen resulting from fixation in the nodules. This is indicated by the increase in ammonia, the first stable intermediate product of symbiotic nitrogen fixation.

Roots of both inoculated and uninoculated nn plants are resistant to nodulation whereas roots of uninoculated NN plants are susceptible to nodulation. Nutman[8] has shown that resistance to further nodulation appears in portions of the root system adjacent to newly formed nodules. For this reason, certain portions of the roots of nodulated NN plants are more susceptible than others to further nodulation. To detect possible metabolic differences, protein, free amino acid, and reducing sugar assays were conducted on fresh root extracts of inoculated and uninoculated NN and nn plants at daily intervals from 5 to 14 days after planting. This time interval was chosen in order to obtain plant material which was uniformly resistant or susceptible to nodulation. Determinations of root dry weight on this

[5] A. J. HOLDING, S. N. TILO and O. N. ALLEN, *Proc. 7th Intern. Congr. Soil Sci.* Vol. 2, Madison, Wisconsin (1960).
[6] J. B. WEIR, *Phyton* **12**, 109 (1960).
[7] D. C. JORDAN, *Bacteriol. Rev.* **26**, 119 (1962).
[8] P. S. NUTMAN, *Ann. Botany* **14**, 79 (1952).

assay material revealed a reduced rate of increase in dry weight in inoculated NN plants as compared with plants of the other three treatments (Fig. 1). This difference, which became apparent about two days before appearance of nodules on inoculated NN plants, may be a result of altered metabolism of NN plants caused by rhizobial infections which are resulting

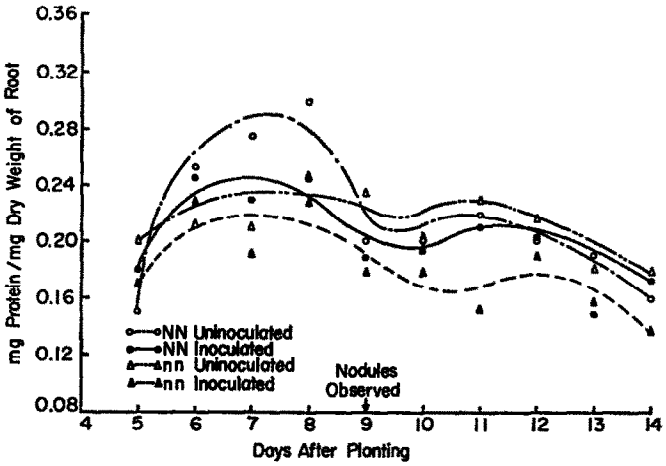

FIG. 2. TOTAL PROTEIN (MG/MG DRY WEIGHT OF ROOT) PER ROOT IN FOUR SOYBEAN TREATMENTS. EACH POINT REPRESENTS THE AVERAGE OF TEN PLANTS.

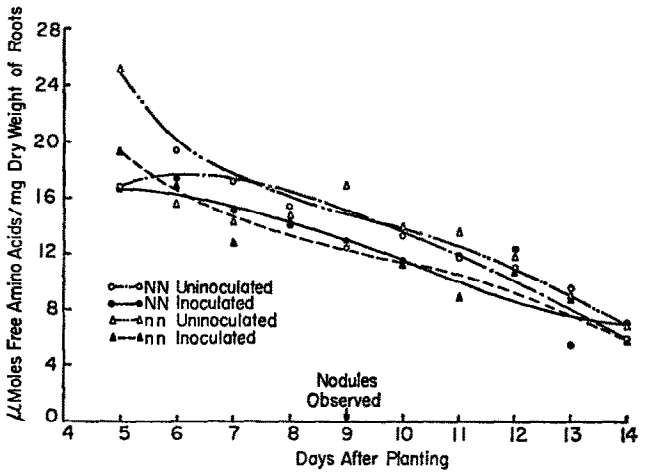

FIG. 3. FREE AMINO ACIDS (μM/MG DRY WEIGHT OF ROOTS) PER ROOT IN FOUR SOYBEAN TREATMENTS. EACH POINT REPRESENTS THE AVERAGE OF TEN PLANTS.

in successful nodule formation. Possible metabolic alterations are increased respiration, decreased synthesis of carbohydrates, or both. Results of assays of these tissues were expressed on the basis of mg dry weight of roots analyzed in order to correct for differences in root mass in the four treatments.

Rate of formation of protein in roots of inoculated NN and nn plants was less than in the corresponding uninoculated plants (Fig. 2). Plant infection apparently results in depression

of protein synthesis in both NN and nn plants. Amounts of protein consistently were lowest in inoculated nn plants, where nodulation fails.

No large differences in amounts of free amino acids were found in roots of the four groups of plants (Fig. 3). On the fifth day after planting, however, the amounts of free amino acids in both groups of nn plants were decreasing from a much higher level than was observed in NN plants. This suggests a much greater and perhaps earlier mobilization of amino acids from cotyledon protein in the nn plant.

Amounts of reducing sugars (Fig. 4) were high in NN plants and low in nn plants before NN plant nodulation. These amounts decreased in inoculated nn plants two days before visible nodulation (of NN plants) but did not drop in inoculated NN plants until the day nodules were observed. The decrease in reducing sugars in inoculated plants indicates a reaction of the plants to rhizobial infection. This reaction occurs earlier and is more pronounced in the inoculated nn plants.

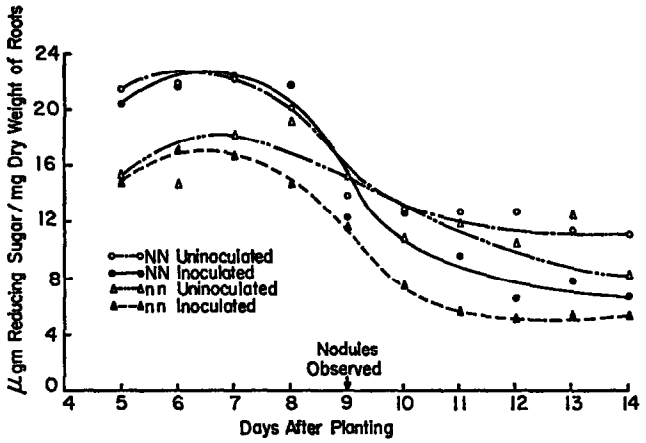

FIG. 4. REDUCING SUGARS (μG/MG DRY WEIGHT OF ROOTS) PER ROOT IN FOUR SOYBEAN TREATMENTS. EACH POINT REPRESENTS THE AVERAGE OF TEN PLANTS.

Wilson[9] found that medium to large amounts of carbohydrate combined with low amounts of nitrogen favored invasion and nodulation while other carbohydrate/nitrogen (C/N) ratios inhibited these processes. C/N ratios calculated from the results of analyses for reducing sugars and free amino acids in the present study are shown in Fig. 5. High C/N ratios were observed initially in nodulation-susceptible NN plants whereas low C/N ratios were observed in nodulation-resistant nn plants. Uninoculated NN plants continued to maintain a high C/N ratio; this indicates continuing susceptibility to nodulation. Nodulation of inoculated NN plants reduces their susceptibility to further nodulation and C/N ratios drop sharply and remain low. This agrees with Nutman's observation that initially formed nodules inhibit further nodulation.[8] Uninoculated nn plants maintain an intermediate C/N ratio and therefore retain some susceptibility to invasion. Inoculated nn plants show a sharp decrease in C/N ratio at the expected time of nodule initiation. This indicates a strong resistant reaction by nn plants which may prevent successful nodule formation. These relationships are consistent with the findings of Wilson.[9]

[9] P. W. WILSON, *Univ. Wis. Agr. Exp. Sta. Res. Bull.* No. 129, Madison (1935).

Very similar amounts of butyric, propionic, acetic, pyruvic, formic and malonic acids were found in all four groups of plants. This indicates at least gross similarity in carbohydrate catabolism in the two plant strains.

The role of phenolic compounds in resistance of plants to invasion by micro-organisms has been discussed by Kuc.[10] The kinds and amounts of phenolic compounds present in the four plant treatments studied here were comparable. Phenolic compounds apparently have no role in inhibition of nodulation in the nn plants either before or after infection.

Canavanine and other guanidoxyl compounds, known to be present in some leguminous plants, could not be detected either in extracts of fresh roots or in hydrolysates of dried root material. This excludes the possibility that such compounds could be acting as specific inhibitors of the invading rhizobia.

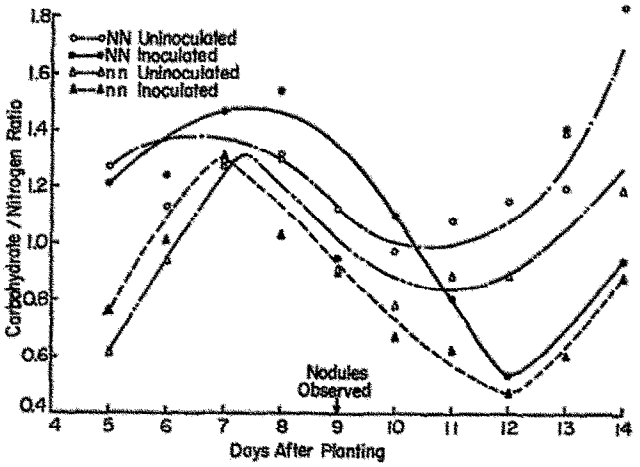

FIG. 5. CARBOHYDRATE/NITROGEN RATIOS PER ROOT OF FOUR SOYBEAN TREATMENTS.

CONCLUSIONS

Analyses of NN and nn soybean seed for major organic and inorganic components revealed no large differences, indicating that early nutrition of the two plant strains may be grossly similar.

Investigation of biochemical patterns in root material of NN and nn plants revealed some definite differences. During the days preceding visible nodulation, roots of uninoculated NN plants contained larger amounts of protein and reducing sugars and smaller amounts of free amino acids than did uninoculated nn roots. Amounts of protein, free amino acids and reducing sugars decreased in inoculated NN and nn plants in comparison with corresponding uninoculated plants, indicating a specific alteration in metabolism by both plant strains in response to rhizobial infection. In the case of free amino acids and reducing sugars this decrease occurred earliest in inoculated nn plants, suggesting a more severe reaction to infection which may be responsible for the ultimate failure of these plants to nodulate.

Carbohydrate/nitrogen (C/N) ratios calculated from the free amino acid and reducing sugar data gave results in agreement with the findings of Wilson.[9] High C/N ratios were associated with plants which were susceptible to nodulation and low C/N ratios were associated with nodulation-resistant plants.

[10] J. KUC, *Conn. Agr. Exp. Sta. Bull.* No. 663, New Haven (1963).

Quantitative metabolic alterations, namely increased respiration and decreased synthesis of carbohydrates, may be responsible for the decreased rate of increase in root dry weight in inoculated NN plants.

MATERIALS AND METHODS

The plant material used in this study came from a cross between a commercial soybean variety (Lee) and a non-nodulating variety (L9-674) developed at the University of Illinois. This cross resulted in a nodulating soybean, NC N 58-5253 (hereafter designated NN), and a segregating, near-isogenic, non-nodulating soybean, NC N 58-5259 (hereafter designated nn).

Mineral analyses of plant material were conducted as described in Official Methods of Analysis of the Association of Official Agricultural Chemists.[11] Seed material was analyzed for fat, carbohydrate, and protein content as follows: Coarsely ground whole seed was dried at 100° for 24 hr and then ground to a fine powder. One g samples were extracted for 24 hr (Soxhlet) with 150 ml of anhydrous diethyl ether. The residue after evaporation of the ether was designated total ether-extractable lipid. The extracted material was re-ground and analyzed for total protein according to the method of Lowry *et al.*[12] Additional samples of 100 mg were hydrolyzed in 100 ml of 2 N HCl and the resulting hydrolysates analyzed for total reducing sugars.[13]

Quantitative analysis of individual amino acids in root material was conducted as follows. Plants were grown in sand flats for fifteen days and watered with Hoagland's N-free nutrient solution[14] and distilled water. Root material was harvested, washed and dried thoroughly. This material was then ground to a fine powder, extracted with ether as above and the residue hydrolyzed and analyzed for amino acids using a Beckman/Spinco Model 120 Amino Acid Analyzer.

Additional analyses of root material were conducted as follows: samples consisting of the roots of ten plants, harvested at daily intervals from 5 to 12 days after planting, were severed above the first lateral root and placed in stoppered test tubes with 10 ml of 95% ethyl alcohol. The root material was macerated with a glass rod and allowed to extract at room temperature for 1 week. One-ml quantities of these alcoholic root extracts were used for quantitative determination of total phenols.[15] Individual phenols were determined by descending paper chromatography after concentrating portions of these extracts by evaporation at 40°. Whatman No. 1 paper was used with either n-butanol:acetic acid:water (6:1:2) or n-amyl alcohol:acetic acid:water (4:1:5) as solvent. After developing the chromatogram for 14 hr, phenolic compounds were detected by scanning under u.v. light and using reagents.[16, 17]

Sugars were determined qualitatively in these same extracts.[18]

Amino acids in root material were determined qualitatively by the method of Mizell and

[11] W. HORWITZ (Editor), *Official and Tentative Methods of Analysis*, Association of Official Agricultural Chemists, Washington, D.C. (1960).
[12] L. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[13] N. NELSON, *J. Biol. Chem.* **153**, 375 (1944).
[14] D. R. HOAGLAND and D. I. ARNON, *Calif. Agr. Exp. Sta. Bull.* No. 347, Berkely (1950).
[15] M. ROSENBLATT and J. V. PELUSO, *J. Assoc. Off. Agr. Chemists* **24**, 170 (1941).
[16] S. SOLOWAY and S. H. WILEN, *Anal. Chem.* **24**, 979 (1952).
[17] R. J. BLOCK, E. L. DURRUM and G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*. Academic Press, New York (1958).
[18] I. C. GUNSALUS, *Experimental Biochemistry*. Stipes, Champaign, Illinois (1959).

Simpson[19] following overnight hydrolysis of dried, finely ground roots in 2 N HCl. The method of Bell[20] was used to test for the presence of canavanine and other guanidoxyl compounds. The pentacyanoammonioferrate color reagent was prepared according to Fearon.[21]

Aqueous root extracts, obtained as indicated below, were used for determination of total protein, organic acids, reducing sugars and amino acids. Seed was planted in vermiculite in pint cardboard containers and plants were watered with distilled water. Appropriate samples were inoculated with a commercial inoculum of *Rhizobium japonicum* (Nodogen Laboratories, Chicago, Illinois) for soybeans. At designated intervals after germination, duplicate ten-plant samples were harvested by washing the plant roots free of vermiculite in ice water. The root systems were cut above the first lateral root and each sample was chopped coarsely and placed in 75 ml of cold 0·1 M NaCl in a Waring Blendor. The sample was blended at high speed for 2 min. Insoluble plant debris was filtered from this extract with suction and washed with 35 ml of 0·1 M NaCl. Dry weight of insoluble root material was determined by drying for 24 hr at 100°.

One-ml portions of the root extract were used to determine total protein[12] and total reducing sugars.[13] An aliquot of this extract was heated on a boiling-water bath for 10 min to precipitate protein. The supernatant resulting after centrifugation was used to determine free amino acids.[22]

Three ml of 10 N NaOH were added to the remaining root extract (approx. 100 ml). This solution was concentrated to about 20 ml on a hot plate, acidified to pH 2·0 with conc. H_2SO_4 and taken up in sufficient silicic acid to make a moist powder. The powder was placed in an extraction thimble and extracted in a Soxhlet extractor with 150 ml of diethyl ether for 24 hr. Before extraction, 2 ml of 1·0 N NaOH and 23 ml distilled water were added to the ether in the distilling flask. Short chain organic acids were extracted from the root sample by the ether and were then trapped as salts in the alkaline aqueous layer in the flask. The ether and water were evaporated off, leaving the salts of the organic acids as a solid residue in a small beaker. The dried sample was acidified with 1 ml of 50% sulfuric acid and the organic acid content of the root extract was then determined by the method of Ramsey[23] with the following modification. In place of the batch-wise addition of solvents and independent pressure source, a linear-gradient system flowing through a Beckman solution metering pump was used to supply solvent and required pressure simultaneously to the top of the column.

Acknowledgements—The authors are indebted to Dr. C. A. Brim, Department of Crop Science, North Carolina State University, who developed and maintained the soybean varieties used in this study. Mineral analyses were conducted in the Analytical Service Laboratory, Department of Soil Science, under the supervision of Mr. J. R. Piland. Amino acid analyses were supervised by Prof. F. H. Smith, Department of Nutritional Biochemistry. This study was partially supported by Grant GB-4378 of the National Science Foundation, and Public Health Service Research Grant AI-107247-01 from the National Institute of Allergy and Infectious Diseases.

[19] M. MIZELL and S. B. SIMPSON, *J. Chromatog.* **5**, 157 (1961).
[20] E. A. BELL, *Biochem. J.* **70**, 617 (1958).
[21] W. R. FEARON, *Analyst* **71**, 562 (1946).
[22] S. MOORE and E. H. STEIN, *J. Biol. Chem.* **176**, 367 (1948).
[23] H. A. RAMSEY, *J. Dairy Sci.* **46**, 480 (1963).