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COMPARATIVE ASPECTS OF ROOT AND ROOT NODULE SECONDARY METABOLISM IN ALFALFA

C. P. VANCE

U.S. Department of Agriculture, Science and Education Administration, Federal Research, Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN 55108, U.S.A.

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Key Word Index—*Medicago sativa*; Leguminosae; alfalfa; phenolics; indoles; phenylalanine ammonia lyase; indoleacetic acid oxidase; leghemoglobin; bacteroids; nodules; roots.

Abstract—L-phenylalanine ammonia lyase (PAL), indoleacetic acid oxidase (IAA oxidase), *O*-methyltransferase, peroxidase, total phenolics and total indoles were compared in roots and root nodules of alfalfa. PAL, *O*-methyltransferase, total phenolics and total indoles were higher in nodules than in roots. Isolated bacteroids were assayed for *O*-methyltransferase, PAL, peroxidase and total phenolics, but their levels were either low or not detectable. Nodule leghemoglobin was separated by disc gel electrophoresis and found to have IAA oxidase activity. Phenolics, IAA oxidase and leghemoglobin appear to be interrelated in regulating indole levels in the nodule.

INTRODUCTION

Secondary metabolites derived from phenylalanine and tryptophan are important in plant growth and development. The role of IAA (derived from tryptophan) is well documented [1, 2]. The effect of phenolics (derived from phenylalanine) on regulation of IAA and IAA oxidase is also well documented [1]. Many studies have compared the metabolism of phenylalanine and tryptophan in plants and various plant organs [2, 3]. A paucity of information, however, exists regarding the comparative biochemistry of these secondary metabolites in root and nodule tissue of legumes, particularly in alfalfa (*Medicago sativa* L.).

Previous comparisons of root and nodule metabolism in legumes have dealt primarily with differences in growth regulating compounds. Pate [4] reported that the IAA content of pea nodules was higher than that in roots. In *Lupinus luteus*, Dullaart [5, 6] reported that IAA was produced from tryptophan and the concentration was substantially higher in nodules than in roots. He also reported that roots and nodules differed in their capacity to degrade IAA, which he attributed to inhibitors in the nodule. In further studies with *Lupinus*, Mennes [7] showed higher IAA oxidase in roots than in nodules. He suggested this difference was the result of differences in phenolic inhibitors in nodules compared to roots. Puppo and Rigaud [8] indicated that a portion of the IAA oxidase activity of soybean nodules may reside in leghemoglobin. These observations suggest that IAA, phenolics and perhaps leghemoglobin may be closely interrelated in the nodule.

The need for further investigations into the metabolism of products derived from phenylalanine and tryptophan in the root and nodule is evident. The studies reported here were undertaken to compare phenylpropanoid and indole metabolism in nodule tissue with that of root tissue of alfalfa.

RESULTS AND DISCUSSION

PAL, peroxidase activities and total phenolics in nodule, root and bacteroid tissue extracts are summarized in Table 1. Nodule tissue contained 120% more total phenolics than root tissue. Phenolics could not be detected in bacteroid tissue. PAL activity was 70% greater in nodules than in roots, while there was no detectable PAL activity in bacteroid tissue extracts. Peroxidase activity was 32% less in nodules than in roots. There was a low but detectable peroxidase activity in bacteroids. Levels of *O*-methyltransferase activity in nodule tissue were significantly greater than those in root tissue with all substrates (Table 2). Caffeic acid was the most efficient substrate. Daidzein and genistein were also methylated more readily by nodule preparations than root preparations but low levels of activity were obtained. Protocatechuic acid was methylated by all preparations. In contrast, bacteroids did not methylate caffeic acid or the two isoflavanoids tested.

These data indicate that metabolism of phenylalanine to phenylpropanoid derivatives was greater in the nodule than in the root tissue. The observed stimulation in phenylpropanoid metabolism occurred primarily in the host rather than in the bacteroids. This is evidenced by the lack of detectable PAL activity, low peroxidase activity, no detectable *O*-methyltransferase activity for caffeic acid and isoflavonoids and no significant quantity of phenolics in bacteroid tissue. The high level of phenolics observed in nodules may have resulted from both an increase in PAL activity providing phenylpropanoid substrates and a reduction in the oxidation of phenolic compounds as evidenced by a lower nodule peroxidase activity. Similar observations have been made with diseased plant tissues, and several authors [4, 5, 7, 9] have suggested that nodules are analogous to galls caused by *Agrobacterium tumefaciens*.

A comparison of total indoles and IAA oxidase

Table 1. Phenylalanine ammonia lyase, peroxidase and total phenolic content of root, nodule, and bacteroid tissue of *Medicago sativa*

Tissue	PAL* ηM cinnamic acid/hr/mg protein	Peroxidase* ΔA/min/mg protein	Phenols*† μg equivalents/g fr. wt.
Nodule	36.2 ± 4.8	34.8 ± 3.0	2112 ± 144
Root	21.1 ± 2.9	51.2 ± 5.7	936 ± 78
Bacteroid	N.D.‡	5.7 ± 2.4	N.D.

* Values represent the average of 4 experiments with 3 replicates in each experiment with S.E.
† Phenols measured as equivalents of *p*-coumaric acid.
‡ Not detectable.

Table 2. *O*-methyltransferase activity in root, nodule and bacteroid tissue of *Medicago sativa*

Tissue	ηM product/hr/mg protein*‡ Ferulic acid	Vanillic acid	Formononetin	Biochanin A
Nodule	244.0 ± 36.3	19.0 ± 3.2	6.8 ± 1.9	8.8 ± 0.8
Root	109.0 ± 32.2	8.0 ± 2.7	2.6 ± 1.2	3.4 ± 1.1
Bacteroid	N.D.†	14.0 ± 3.0	N.D.	N.D.

* Values are the average of 5 experiments with 3 replicates in each experiment with S.E.
† Not detectable.
‡ Substrate for each respective product was caffeic acid, protocatechuic acid, daidzein, genistein.

activity showed that total indoles were higher in the nodules than in the roots (Table 3). It should be noted that the indole content of the nodule was not just IAA since the procedure used would measure both indole precursor and degradation products of IAA. IAA oxidase activity in the nodule was 33% that of root tissue.

IAA has been shown to be higher in the nodules than in the roots of both pea and lupin [6, 4]. IAA concentration in root nodules of lupin was 3 times higher than that in roots [6], while in pea IAA was 50 times higher in nodules than in roots [4]. The values for indole concentration in alfalfa in this study are similar to the results reported for pea. Mennes [7] showed lupin to have significantly lower IAA oxidase activity in the nodule than in the root, and ascribed this to endogenous inhibitors, probably phenolic. The data presented in this report agree with Mennes' suggestion.

The reported high levels of IAA in nodules may result from increased synthesis accompanied by reduced degradation because of lower IAA oxidase activity [5–7]. Phenolics, particularly caffeic and ferulic acids, are considered potent inhibitors of IAA oxidase activity [1]. In alfalfa, high phenolics in the nodule were accompanied by high *O*-methyltransferase activity (Tables 1 and 2). The most effective substrate for this enzyme was caffeic acid which was converted to ferulic acid. It would appear that the increased capacity of the nodule to synthesize ferulic acid or related compounds may prevent IAA degradation by inhibiting IAA oxidase activity.

Table 3. Indoles and IAA oxidase activity in root and nodule tissue of *Medicago sativa*

Tissue	Indoles*† μg equivalents/g fr. wt	IAA oxidase* μM IAA destroyed/mg protein
Nodule	8.00 ± 3.1	0.69 ± 0.22
Root	0.24 ± 0.2	2.20 ± 0.56

* Values represent the average of at least 4 experiments with 3 replicates in each experiment with S.E.
† Indoles measured as equivalents of indole-3-acetic acid.

Preliminary studies of IAA oxidase in root and nodule tissue of alfalfa indicated that a portion of the nodule IAA oxidase activity was associated with the leghemoglobin fraction. To further verify this the leghemoglobin fraction of nodule tissue extracts was precipitated with $(NH_4)_2SO_4$ (0.65–0.80) saturation, and further purified by disc gel electrophoresis. The gel scans indicated 3 major and 3 minor peaks for leghemoglobin as measured by absorbance at 425 nm. IAA oxidase activity was associated with the 3 major leghemoglobin peaks and the major peak corresponded to the leghemoglobin peak at 3.5 cm. The minor leghemoglobin peak at 6.0 cm did not appear consistently and had no IAA oxidase activity. The increase in absorbance at 530 nm beyond 6.0 cm resulted from the diffusion of the tracking dye into the gel during enzyme assay. Also another IAA oxidase peak was found at 9.4 cm that did not correspond to leghemoglobin. All areas in the gel that showed IAA oxidase activity also showed peroxidase activity with pyrogallol.

Leghemoglobin is thought to function by regulating oxygen tension in the nodule [9, 10]. The data presented here indicate that leghemoglobin may also act as an IAA oxidase accompanied by a peroxidase function with pyrogallol. This observation agrees with the results of Puppo and Rigaud [8] who showed that soybean leghemoglobin, in the oxidized state, acted as an IAA oxidase. Nitrite has been shown to oxidize leghemoglobin [11]. Nodules of alfalfa have a high nitrate reductase activity (Vance, unpublished). These observations suggest that in the presence of high nitrate concentrations leghemoglobin may function as an IAA oxidase as well as to regulate oxygen tension in the nodule.

IAA has been implicated as a major compound associated with nodule morphogenesis and growth [4, 5, 10]. The results presented in this report indicate that phenolic metabolism, IAA oxidase and leghemoglobin are all interrelated in regulation of IAA levels in alfalfa nodule tissue.

EXPERIMENTAL

Plant material. Field-grown alfalfa (*Medicago sativa* L. Apollo) plants were dug 60–65 days after emerging from the soil. Nodules and roots were separated by hand and either stored on ice for immediate use or frozen and stored at −20° for later use.

Preparation of cell-free extracts. Cell-free extracts of roots and nodules were prepared by grinding the tissue in acid washed sand with Polyclar AT (0.1 g/g tissue) and extracting twice with 0.05 M Na Pi buffer (2 ml/g tissue) pH 7.0, containing 10 mM mercaptoethanol. The resultant slurry was centrifuged at 400 *g* for 15 min and the pellet discarded. Bacteroids were collected from the nodule supernatant by a second centrifugation at 6800 *g* for 20 min. To further clarify the supernatants from nodules and roots, the prepns were centrifuged at 15000 *g* for 20 min and the pellet was discarded. The supernatant was treated with solid $(NH_4)_2SO_4$ to 0.80 saturation. The ppt. formed in 45 min was collected by centrifugation at 15000 *g* for 15 min. The ppt. was dissolved in a small vol. of 0.05 M Na Pi buffer, pH 7.0. The prepn was desalted by dialysis or by passage through Sephadex G-25 (coarse grade).

Bacteroids were homogenized in a Braun Homogenizer with 0.25–0.33 mm glass beads in the same buffer as used for plant material (3 ml/g bacteroids). The homogenate was centrifuged at 15000 *g* for 20 min and the pellet was discarded.

The pink-red supernatant from nodules also was the source of leghemoglobin. A portion of the supernatant was treated with solid $(NH_4)_2\ SO_4$ and the fraction that pptd between 0.65–0.80 saturation was collected for leghemoglobin. The ppt. was dissolved in a minimal amount of 0.05 M Na Pi buffer at pH 6.5. The concd leghemoglobin was dialysed against the same buffer for 16 hr.

Enzyme assays and electrophoresis. PAL activity was measured by the procedures of refs. [12, 13]. *O*-methyltransferase was assayed according to the procedure ref. [14]. The assay for peroxidase was that described by ref. [15]. IAA oxidase was measured according to the procedure of ref. [16]. The radioactive products were identified by cochromatography with authentic standards.

The electrophoresis procedure for leghemoglobin was that described by ref. [17]. After electrophoresis gels were scanned at 425 nm and 563 nm for leghemoglobin. The gels were stained for IAA oxidase according to ref. [18] or peroxidase according to ref. [19]. After the gels were stained for enzyme activity and scanned they were compared with gels containing leghemoglobin.

Measurement of total phenolics and indoles. Phenolics were measured in tissue according to the procedure described by ref. [20] with *p*-coumaric acid as the standard. Indoles were measured according to ref. [21], with IAA as the standard.

General methods. Protein was measured according to ref. [22]. Radioactivity in products from enzyme assays was determined in a scintillation cocktail containing 4 g Omniflour (New England Nuclear) in 750 ml toluene and 250 ml 95% EtOH. Spectra and gel scans were obtained on a Spectrophotometer with gel scan accessory.

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