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Root and Nodule Enzymes of Ammonia Assimilation in Two Plant-Conditioned Symbiotically Ineffective Genotypes of Alfalfa (*Medicago sativa* L.)[1]

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ABSTRACT

Biochemical and physiological parameters associated with nitrogen metabolism were measured in nodules and roots of glasshouse-grown clones of two symbiotically ineffective alfalfa (*Medicago sativa* L.) genotypes supplied with either NO_3^- or NH_4^+. Significant differences were observed between genotypes for nodule soluble protein concentrations and glutamine synthetase (GS) and glutamate synthase (GOGAT) specific activities, both in untreated controls and in response to applied N. Nodule soluble protein of both genotypes declined in response to applied N, while nodule GS, GOGAT, and glutamate dehydrogenase (GDH) specific activities either decreased or remained relatively constant. In contrast, no genotype differences were observed in roots for soluble protein concentrations and GS, GOGAT, and GDH specific activities, either in untreated controls or in response to applied N. Root soluble protein levels and GS and GOGAT specific activities of N-treated plants increased 2- to 4-fold within 4 days and then decreased between days 13 and 24. Root GDH specific activity of NH_4^+-treated plants increased steadily throughout the experiment and was 50 times greater than root GS or GOGAT specific activities by day 24.

Enzymological data indicate that nodules of these ineffective alfalfa genotypes are uniquely differentiated plant organs. Decreasing or constant plant GS and GOGAT specific activities in ineffective nodules in response to applied N suggest that factors in addition to N supply are involved in the induction of high levels of plant ammonia-assimilating enzymes in nodules. Genotype differences observed for nodule enzyme specific activities support the concept that ineffectiveness may be expressed in different ways within the nodule. Senescence was evident in ineffective nodules of N-treated plants of both genotypes, indicating that nodule senescence induced by applied N may not be closely linked to symbiotic effectiveness in alfalfa. Data for ammonia-assimilating enzymes in roots suggest the GS/GOGAT pathway operates only at low levels of soil N and that GDH functions to detoxify high levels of soil NH_4^+.

The development of an effective N_2-fixing symbiosis involves complex interaction between host plant and *Rhizobium* symbiont (1, 9, 18). Alterations in either genome and the environment can result in an ineffective association (1, 7, 10). Strains of *Rhizobium* that induce ineffective nodules on normally effective host plants have been reported for a number of legumes. The structure and physiology of these *Rhizobium*-conditioned ineffective nodules have been studied extensively (1, 3, 8–10, 14–17). To our knowledge, only three studies have examined N assimilation in *Rhizobium*-conditioned ineffective nodules. Werner *et al.* (16), Sen and Schulman (14), and Duke and Ham (3) reported decreased specific activities of plant GS,[3] GOGAT, and GDH in soybean nodules induced by an ineffective strain of *R. japonicum* compared to soybean nodules induced by an effective strain. In contrast, relatively few studies have examined plant-conditioned ineffective nodules and these have been structural studies (1, 17–19). Viands *et al.* (19) recently reported that plant-conditioned ineffective nodules of the alfalfa genotype MnPL-480 contained no leghemoglobin, but starch concentration and nitrate reductase activity were higher than in effective nodules. Comparative physiological and biochemical studies of plant-conditioned ineffective nodules, particularly with respect to N assimilation, offer potential for understanding the contribution of the host plant to symbiotic effectiveness. Such studies, however, have been neglected due to lack of available plant genetic material.

Several plant-conditioned ineffective genotypes have been discovered during the course of alfalfa breeding programs at Minnesota. These genotypes produce ineffective nodules with strains of *R. meliloti* that form nodules on normal alfalfa cultivars and these plants require applied NO_3^- or NH_4^+ for growth. Recent reports have described the genetics (11) and developmental nodule anatomy (6, 17, 18) of these ineffective traits in alfalfa. The data indicate that a number of plant genes regulate nodule structure and function.

Nodules of these ineffective alfalfa genotypes are of two basic morphological types (18). One type (MnPL-480) appears gall- or tumor-like and contains few if any bacteroids, while the other type [MnSa(In)] is more similar in structure to effective nodules, but their bacteroids senesce early in development (6, 17, 18). Since these nodules are ineffective due to host genetics factors, studies of N assimilation in both genotypes will be useful for assessing plant factors associated with and regulating N_2 fixation. Since nodule growth and development is occurring with little if any N_2 fixation and/or bacteroid development, these ineffective genotypes may be useful in evaluating the photosynthetic requirements for maintenance of nodule structure without interference of the symbiont. Because of the potential these genotypes offer, it is particularly important to investigate the physiology and biochemistry of plant-conditioned ineffective nodules.

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[3] Abbreviations: GS, NH_3-dependent glutamine synthetase; GOGAT, NADH-dependent glutamate synthase; GDH, NADH-dependent glutamate dehydrogenase (reductive amination).

Earlier studies of effective nodules in our laboratory showed ammonia assimilation occurred via the GOGAT/GS pathway and that GDH may be important during senescence (5). Applied N induced senescence and reduced GS, GOGAT, and soluble protein in actively fixing effective nodules. Our studies and several others (3, 13, 14, 16), have been unable to define clearly the role of the host plant and symbiont in regulating nodule N metabolism. We thought studies of N assimilation in plant-conditioned ineffective genotypes may delineate these roles. The objectives of this study were to measure plant enzymes of ammonia assimilation in nodules and roots of two plant-conditioned ineffective alfalfa genotypes, representative of the two ineffective nodule morphologies grown in the presence of either NO_3^- or NH_4^+.

MATERIALS AND METHODS

Plant Material. Ineffective alfalfa (*Medicago sativa* L.) clones MnPL-480 and MnSa(In) were propagated by means of stem cuttings. Propagules were grown in pots of sand, inoculated with *R. meliloti* (Nitragin Co., Milwaukee, WI,[4] and G. Hardarson, University of Minnesota) and supplemented with micro- and macronutrients (except for N) at regular intervals. Plants were grown for 12 months in a glasshouse with supplemental fluorescent light (350 $\mu E\ m^{-2}\ s^{-1}$) during a 16/8 h light/dark cycle at 25/20°C. Minimal amounts (25 mg) of N as NO_3^- were supplied when N deficiency symptoms became apparent and plant tops were harvested (80% shoot removal) regularly at flowering. Three weeks prior to the beginning of the experiment, plants were repotted in fresh sand supplemented with micro- and macronutrients (except for N), and plant tops were harvested. At the beginning of the experiment (day 0) and on even-numbered days thereafter, N either as KNO_3 or as $(NH_4)_2SO_4$ was applied as an aqueous solution to the sand to give a concentration of 50 $\mu g/g$ N, and control plants were treated with an equivalent amount of K_2SO_4. Treatments were applied in a completely randomized design. Three plants of each genotype-treatment were sampled near 8:30 AM on days 0, 1, 4, 13, and 24. Nodules were picked and fibrous roots were excised and placed in beakers on ice until extraction. Effective plants were grown as previously described (5).

Preparation of Cell-Free Extracts. Cell-free extracts of nodule and root samplings were prepared at 0 to 4°C using an extraction buffer consisting of 100 mM Mes-NaOH (pH 6.8), 100 mM sucrose, 2% (v:v) 2-mercaptoethanol, and 15% (v:v) ethylene glycol as previously described (5).

Enzyme Assays. Ammonia-dependent GS activity in cell-free extracts was determined using a radioisotopic assay (12). Glutamate synthase and GDH were measured spectrophotometrically, monitoring absorbance due to NADH at 340 nm. Conditions for enzyme assays were those we have described previously for effective alfalfa nodules (5). Acetylene reduction was measured as described previously (19).

Protein Determination. Soluble protein was measured by the method of Bradford (2) using a reagent consisting of 0.2% Coomassie Brilliant Blue G-Type in 3.5% $HClO_4$. Standard curves were prepared using BSA (20 to 250 μg protein).

RESULTS

Nodule Parameters. Nodule soluble protein (Fig. 1) decreased in response to applied N by day 13 and continued to decline through day 24 for both genotypes. Nodule soluble protein levels of control plants were twice as high for MnPL-480 as for MnSa(In). MnPL-480 nodule soluble protein was significantly less for NH_4^+- than for NO_3^--treated plants on days 13 and 24. In contrast, nodule soluble protein of MnSa(In) was slightly greater for NH_4^+-treated plants at these times.

MnPL-480 nodule GS specific activity was minimal and was not affected by applied N. In contrast, nodules of MnSa(In) exhibited appreciable GS specific activity which declined significantly and progressively in response to both forms of applied N.

Nodule GOGAT specific activity of MnPL-480 plants was very low and did not respond to applied N. MnSa(In) nodule GOGAT

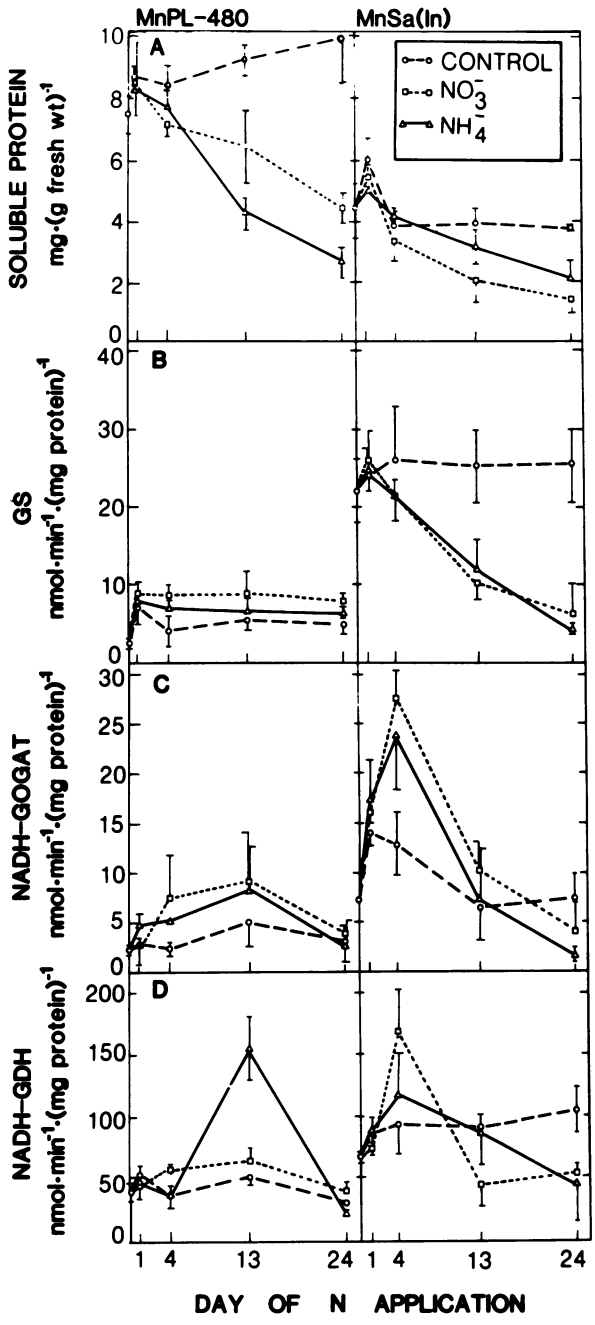

FIG. 1. Nodule soluble protein, GS, GOGAT, and GDH activities in ineffective alfalfa genotypes MnPL-480 (left) and MnSa(In) (right). Beginning on day 0 and on successive even-numbered days, nitrogen as either KNO_3 or $(NH_4)_2SO_4$ was applied to pots to give a final concentration of 50 $\mu g/g$ N. Control plants were treated with equivalent amounts of K_2SO_4. Each point is the mean of three replicates ± SE.

[4] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by either the U. S. Department of Agriculture or University of Minnesota and does not imply its approval to the exclusion of other products that may also be suitable.

specific activity increased on day 4 but then declined to control levels by day 13 in response to applied N. Nodule GOGAT specific activity of MnSa(In) was higher than MnPL-480 through day 4 for a given N treatment but did not differ between genotypes thereafter. There was no significant difference between NO_3^- and NH_4^+ treatments for nodule GOGAT specific activity of either genotype.

Applied N had relatively little effect on nodule GDH specific activity of either MnPL-480 or MnSa(In) alfalfa, except for NH_4^+-treated MnPL-480 plants on day 13. Nodule GDH specific activity of control plants was twice as high for MnSa(In) than for MnPL-480.

Root Parameters. Applied N increased root soluble protein of both genotypes by day 4 (Fig. 2). Root soluble protein of N-treated MnPL-480 plants remained twice as high as controls for the remainder of the experiment. MnSa(In) root soluble protein decreased slightly in response to applied NH_4^+ between days 13 and 24. Control levels of MnSa(In) root soluble protein increased slightly throughout the experiment compared to MnPL-480 controls. Nitrate-treated plants of both genotypes had root soluble protein levels intermediate between those of NH_4^+-treated and control plants on days 4 and 13. Root soluble protein of NO_3^-- and NH_4^+-treated plants of both genotypes were similar on day 24.

Root GS specific activity of N-treated plants of both genotypes increased steadily through day 4, remained high through day 13, and then decreased to control levels by day 24. Root GS specific activity of NH_4^+-treated plants was greater for MnPL-480 than for MnSa(In) on days 4 and 13, but there was no difference between genotypes for control and NO_3^--treated plants at any time. Root GS specific activity of NO_3^-- and NH_4^+-treated MnPL-480 plants did not differ on days 4 and 13, whereas MnSa(In) root GS specific activity was greater for NO_3^-- than for NH_4^+-treated plants at these times. On day 24, root GS specific activity of both genotypes was less for NH_4^+- than for NO_3^--treated plants.

Root GOGAT specific activity of both MnPL-480 and MnSa(In) followed a pattern similar to that of root GS in response to applied N, increasing initially and then decreasing between days 13 and 24. In contrast to root GS specific activity, however, root GOGAT specific activity of N-treated plants remained higher than controls on day 24. There was no significant difference between genotypes in root GOGAT specific activity, except that of NO_3^--treated plants was less for MnSa(In) than for MnPL-480 on day 13. Applied NO_3^- and NH_4^+ had similar effects on root GOGAT specific activity throughout the experiment.

Applied N increased root GDH specific activity of both genotypes by day 4. Specific activity of NH_4^+-treated plants continued to increase steadily throughout the experiment, reaching levels 50 times higher than controls by day 24. Root GDH specific activity of NO_3^--treated plants increased to 10 to 20 times that of controls after 4 days and then remained constant through day 24. There was no difference between genotypes at any sampling for a given N treatment.

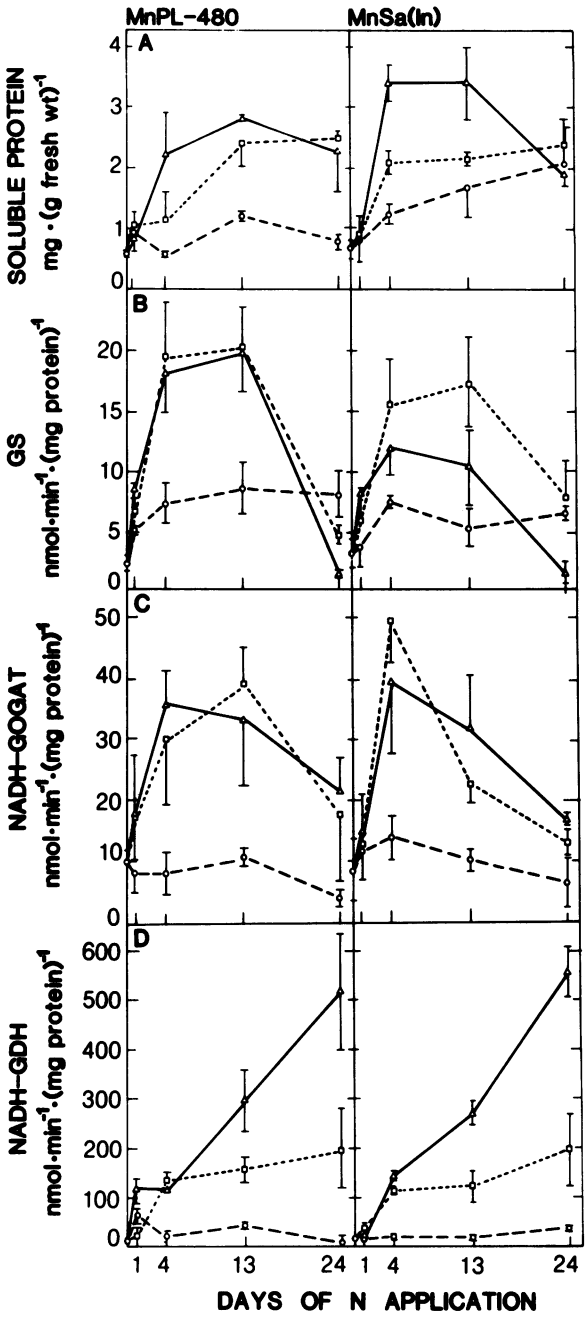

FIG. 2. Root soluble protein, GS, GOGAT, and GDH activities in ineffective alfalfa genotypes MnPL-480 (left) and MnSa(In) (right). Beginning on day 0 and on successive even-numbered days, nitrogen as either KNO_3 or $(NH_4)_2SO_4$ was applied to pots to give a final concentration of 50 μg/g N. Control plants were treated with equivalent amounts of K_2SO_4. Each point is the mean of three replicates ± SE.

DISCUSSION

Nodules that form on ineffective alfalfa genotypes differ from each other and from effective nodules in many biochemical parameters (Table I). MnSa(In) nodules have less soluble protein than either MnPL-480 or effective Saranac nodules, however, nodule GS and GOGAT of MnSa(In) are intermediate to MnPL-480 and effective Saranac. The response of MnSa(In) nodule GS and GOGAT to applied N is similar to that we reported in an earlier study of effective nodules (5). In contrast, GS and GOGAT specific activities in MnPL-480 nodules were relatively unaffected by applied N. MnSa(In) plants produced small but significant amounts of ethylene when incubated for 3 h in 10% acetylene, while MnPL-480 produced no detectable ethylene (Table I). These data suggest MnSa(In) nodules may be slightly effective, whereas MnPL-480 nodules appear to be completely ineffective. Biochemical and physiological differences between MnSa(In) and MnPL-480 may reflect reported differences in nodule structure and development (6, 17, 18). Previous studies showed MnSa(In) nodules to be similar in structure and development to normal effective nodules, but bacteroids remained small and nodules senesced prematurely. In contrast, MnPL-480 nodules are large, tumor-like, contain no leghemoglobin, have few cells containing bacteroids, and are filled with starch. The differences between MnSa(In) and MnPL-480 reported here and in earlier studies substantiate the

Table I. *Comparisons of Acetylene Reduction, Nodule Soluble Protein and Ammonia-Assimilating Enzymes between Effective Saranac and Ineffective MnPL-480 and MnSa(In) Alfalfa Genotypes*

Nodules were collected from 6-month-old MnPL-480 and MnSa(In) plants maintained on minimal N while effective nodules were collected from Saranac plants of comparable age grown on zero N.

Genotype	Acetylene Reduction	Soluble Protein	GS	GOGAT	GDH
	% effective control[a]	*mg g^{-1} nodule fresh wt*		*nmol min^{-1} mg^{-1} protein*	
Saranac	100	21 ± 3.0[b]	85 ± 9	90 ± 11	65 ± 5
MnSa(In)	2	3 ± 0.7	25 ± 4	18 ± 2	84 ± 12
MnPL-480	0	9 ± 1.0	16 ± 4	5 ± 1	56 ± 6

[a] Acetylene reduction based on a 3-h incubation period.
[b] Data given as $\bar{X}$ ± SE for three replicates.

hypothesis (8, 9, 18) that there may be varying degrees or types of ineffectiveness and that ineffectiveness may be controlled by several genes (11).

Specific activities of ammonia-assimilating enzymes in untreated controls expressed as nmol min^{-1} mg protein^{-1} were GS-25, GOGAT-18, and GDH-84 in MnSa(In) nodules, while specific activities in MnPL-480 nodules were GS-16, GOGAT-5, and GDH-56. In comparison, effective alfalfa nodules showed GS, GOGAT, and GDH specific activities to be 85, 90, and 65 nmol min^{-1} mg protein^{-1}, respectively (Table I). We observed similar activities for these ammonia-assimilating enzymes in effective nodules in a previous study (5). Nodules GS and GOGAT have been implicated in plant assimilation of fixed N, but the role of nodule GDH remains obscure (4, 13). Data from this study provide further evidence that alfalfa nodule GS and GOGAT function to assimilate symbiotically-fixed N and that nodule GDH is not associated with active N_2 fixation. Decreasing or constant GS and GOGAT specific activities in ineffective nodules in response to applied N suggest that factors other than N supply are involved in the induction of high levels of nodule ammonia-assimilating enzymes. GS and GOGAT specific activities of MnSa(In) ineffective nodules did not respond in a coordinated fashion to applied N. Similar results have been reported for effective alfalfa nodules (5), indicating that nodule GS and GOGAT activities are not always tightly coupled. Werner *et al.* (16) reported decreased levels of plant GS and GDH for bacterial-conditioned ineffective soybean nodules. Specific activities of other enzymes were greater in ineffective nodules, suggesting these bacterial-conditioned ineffective soybean nodules are specifically differentiated and not just generally reduced in metabolic activities compared to effective nodules. Our biochemical data, as well as histological observations of nodule development for various ineffective associations between alfalfa and *R. meliloti* (6, 15, 17, 18), support this idea and suggest that it can be extended to nodules that are ineffective due to host plant genetic factors.

The roots of these two alfalfa clones did not differ in soluble protein concentration, GS, GOGAT, and GDH specific activities either in untreated controls or in response to applied N. Roots of normal effective alfalfa genotypes had soluble protein levels and GS, GOGAT, and GDH specific activities (R. G. Groat and C. P. Vance, unpublished data) similar to MnPL-480 and MnSa(In) roots. Thus, the differences observed in nodules for these biochemical parameters are not due to corresponding differences in roots. The differences in these parameters observed between roots and ineffective nodules demonstrate that they are uniquely differentiated plant organs. Nodules of these ineffective alfalfa genotypes are therefore not simply hypertrophic or hyperplastic root tissue.

The response of root GS and GOGAT specific activities to applied N suggests that root GS and GOGAT may participate in ammonia assimilation at low levels of soil N and that these root enzymes cease to function at high soil N levels. Root GDH data are consistent with this enzyme functioning to detoxify high levels of soil NH_4^+ as suggested previously (3, 4).

Soluble protein concentrations in ineffective nodules of both clones declined in response to applied N. Similar results have been reported for senescing effective alfalfa nodules (5). There was also morphological evidence of nodule senescence in response to applied N for both ineffective genotypes as nodules of N-treated plants became brown and shriveled in appearance. In contrast to ineffective nodules of these genotypes, soluble protein in roots increased initially in response to applied N. Root soluble protein of NH_4^+-treated plants later declined, suggesting levels of soil NH_4^+ toxic to root tissue may have been reached by day 24. Root and nodule tissues appear to differ greatly in sensitivity to combined N. Our results indicate the mechanism of N-induced nodule senescence is not closely linked to active N_2 fixation in alfalfa. It may be possible to manipulate genetically nodule sensitivity to soil N and symbiotic effectiveness independently.

MnPL-480 and MnSa(In) along with several other ineffective alfalfa genotypes discovered at Minnesota, are potentially useful tools for studying symbiotic N_2-fixation. Ineffective plant genotypes may have practical application for field studies of N_2-fixing legumes, inasmuch as these genotypes form nodules with normally effective strains of *Rhizobium* but do not fix significant N_2. Comparative physiological and biochemical investigations of plant-conditioned ineffectiveness should be useful for assessing host plant factors involved in effective legume-*Rhizobium* associations.

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