Host Plant Cultivar Effects on Hydrogen Evolution by *Rhizobium leguminosarum*

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ABSTRACT

The effect of host plant cultivar on *H₂* evolution by root nodules was examined in symbioses between *Pisum sativum* L. and selected strains of *Rhizobium leguminosarum*. Hydrogen evolution from root nodules containing *Rhizobium* represents the sum of *H₂* produced by the nitrogenase enzyme complex and *H₂* oxidized by any uptake hydrogenase present in those bacterial cells. Relative efficiency (RE) calculated as RE = 1 - (H₂ evolved in air/3H₂ reduced) did not vary significantly among 'Feltham First', 'Alaska', and 'J11205' peas inoculated with *R. leguminosarum* strain J11205, which lacks uptake hydrogenase activity (Hup⁻). That observation suggests that the three host cultivars had no effect on *H₂* production by nitrogenase. However, RE of strain 128C53 was significantly (P ≤ 0.05) greater in symbiosis with cultivar J11205 than in root nodules of Feltham First. At a similar rate of *C₃H₂* reduction on a whole-plant basis, nearly 24 times more *H₂* was evolved from the Feltham First/128C53 symbiosis than from the J11205/128C53 association. Root nodules from the Alaska/128C53 symbiosis had an intermediate RE over the entire study period, which extended from 21 to 36 days after planting. Direct assays of uptake hydrogenase by two methods showed significant (P ≤ 0.05) host cultivar effects on *H₂* uptake capacity of both strain 128C53 and the genetically related strain J960. The *3H₂* incorporation assay showed that strains 128C53 and J960 in symbiosis with Feltham First had about 10% of the uptake hydrogenase activity measured in root nodules of Alaska or J11205. These data are the first demonstration of significant host plant effects on rhizobial uptake hydrogenase in a single plant species.

Hydrogen evolution from leguminous root nodules represents the sum of *H₂* formation by nitrogenase and *H₂* oxidation by any uptake hydrogenase present within the *Rhizobium* cells. No plant enzyme system which metabolizes *H₂* has been identified in root nodule cells. Bulen and LeComte (5) first demonstrated an ATP-dependent formation of *H₂* by a bacterial nitrogenase enzyme complex, and it is now known that, various biochemical factors can influence such *H₂* formation (19). Hydrogen uptake by leguminous root nodules was first reported in the pea system in 1941 (25). That work was confirmed by Dixon (11), who localized uptake hydrogenase activity in pea root nodule bacteroids (12) and found it to be similar to *Azotobacter* hydrogenase in all respects examined (13).

Many data suggest that at least 25% of the reductant used by nitrogenase is allocated to protons for *H₂* formation, while the remaining fraction of reductant is used to convert *N₂* to *NH₃* (6). The EAC is a convenient expression that reflects the partitioning of reductant among protons and alternative substrates such as *N₂* or *C₃H₂* (6): EAC = (exogenous substrate reduced/*H₂O* reduced + exogenous substrate reduced). In the presence of saturating levels of *C₃H₂*, all reductant is used to produce *C₃H₄* and no *H₂* formation is detected. Schubert and Evans (28) defined the relative efficiency of *N₂* fixation as RE = 1 - (H₂ evolved in air/3C₃H₂ reduced) and showed that RE varied greatly among different *Rhizobium*-legume symbioses. Such RE values depend on the EAC of nitrogenase and on the capacity of the rhizobial cells to recover *H₂* by a separate uptake hydrogenase. Thus, in the absence of uptake hydrogenase activity (Hup⁻ phenotype), RE presumably is a measure of apparent EAC.

Previous reports indicate that both plant and bacterial factors can affect *H₂* evolution from leguminous root nodules. Dixon (13) showed that *R. leguminosarum* strain ONA 311 expressed strongly Hup⁺, slightly Hup⁻, or Hup⁻ phenotypes on *Pisum sativum*, *Vicia bengalensis*, and *V. faba*, respectively. Similar reversals in uptake hydrogenase phenotype have been observed for two *R. japonicum* strains associated with *Vigna unguiculata* and *Glycine max* (20). Both strains were Hup⁺ on three cowpea cultivars and Hup⁻ on three soybean cultivars. Host plant effects on apparent rhizobial EAC have been indicated by the observation that lengthening the normal dark period for *P. sativum* and *Trifolium subterraneum* increased RE of Hup⁻ *Rhizobium* symbionts (15). Host plant age and long-term environmental factors such as availability of combined nitrogen, irradiance, and *CO₂* level also can affect apparent EAC (16). These latter findings are consistent with a previous study which concluded that both EAC and uptake hydrogenase activity of a Hup⁺ rhizobial strain were affected by irradiance treatment of the host pea plant (3). Effects of bacterial genotype were evident from the report that only two of four *R. leguminosarum* strains tested were Hup⁺ in pea root nodules (13). More extensive, subsequent surveys reported that six of 15 *R. leguminosarum* strains tested on 'Australian Winter' peas had uptake hydrogenase activity (27) and that only 14 of 79 effective *R. leguminosarum* isolates tested on 'Homesteader' peas had significant uptake hydrogenase activity (24). No workers studying *R. leguminosarum* have reported uptake hydrogenase activity which was sufficient to recover all *H₂* evolved from nitrogenase.

One *Rhizobium* strain of particular interest, which deserves further study, is *R. leguminosarum* 128C53. This strain has been variously described in the literature as exhibiting Hup⁺ (3, 23, 24, 26) and Hup⁻ (14) phenotypes on *P. sativum*. Each of the groups studying strain 128C53 used a different pea cultivar as a host.

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4 Abbreviations: EAC, electron allocation coefficient of nitrogenase; RE, relative efficiency of nitrogenase; Hup⁺, hydrogen-uptake positive; Hup⁻, hydrogen-uptake minus.
plant, and it is impossible to evaluate the apparently conflicting reports. The genetic determinants responsible for uptake hydrogenase activity in 128C53 have been transferred by conjugation to other strains (4) and were part of a recombinant plasmid which was used to construct a Hup+ strain that was symbiotically superior to both parent strains (8). If the genetic determinants responsible for the Hup+ phenotype are influenced by the host plant genotype, as the one contradictory report (14) seems to imply, then it is important to verify and examine that fact. The immediate goal of our study was to test whether strain 128C53 expressed Hup+ and Hup− phenotypes on different cultivars of *P. sativum*. A clear demonstration of such a phenomenon could form the basis for future physiological and genetic studies of host plant factors affecting H2 evolution by *Rhizobium*. Such studies are not feasible in systems where different species of legumes are required to elicit varying phenotypes in symbiotic rhizobial cells.

**MATERIALS AND METHODS**

*Plants.* *Pisum sativum* L. cv Alaska (Burpee Seed, Riverside, CA), Feltham First (Finney Lock, Witham, Essex), and J11205 (courtesy of B. Snoad, John Innes Institute, Norwich, Norfolk) were grown in controlled environmental chambers under a 16/8 h light/dark cycle, 21–15°C, 50% RH, and a photosynthetic photon flux density (400–700 nm) of 650 μE·m−2·s−1. Seeds were sterilized, germinated, and selected for uniformity before planting in sterile 750-ml Leonard jars containing vermiculite (10). All plants were provided with sterile N-free nutrient solution (10) and a single strain of *Rhizobium* in the Leonard jar.

*Bacteria.* *Rhizobium leguminosarum* strains 128C53, 300, and 3960 were used. Strains 128C53 and 300 originally were isolated from the field and show Hup+ and Hup− phenotypes, respectively, on Alaska pea (4). Strain 3960 was constructed by transferring determinants for nodulation, N2 fixation, and Hup+ from 128C53 through conjugation to 300 (8). Bacteria were maintained asymptotically on LMB medium (22).

**Physiological Assays.** Nitrogenase activities were measured on detached root systems excised at the cotyledonary node. Hydrogen evolution in air and C2H2-dependent C2H4 production (C2H4

![Graph](https://example.com/graph.png)

**FIG. 1.** Effect of *Pisum sativum* cultivars Feltham First, Alaska, and J11205 on H2 evolution in air and C2H2 reduction by root nodules formed by *R. leguminosarum* 128C53. Hydrogen evolution was measured in the absence of C2H2. The C2H4 reduction values were varied by assaying plants of different ages ranging from 5 d before flowering to 12 d after flowering. Values are the mean ± SE of six replicate plants.

![Graph](https://example.com/graph.png)

**FIG. 2.** Effect of C2H2 concentration on C2H2 reduction and simultaneous H2 evolution by root nodules formed on Feltham First peas by *R. leguminosarum* 128C53. Each value represents the mean ± SE of six replicate plants at flowering. 26 d after planting. Similar saturation characteristics were observed for strain 128C53 on Alaska and J11205 peas at flowering.

Reduction) were assayed sequentially within 30 min after shoot excision, when C2H2 reduction activities were linear. A Perkin-Elmer Sigma IV gas chromatograph equipped with a thermistor detector was operated with a Molecular Sieve 5A (60–80 mesh) column (0.3 × 254 cm) to measure H2. Oven temperature was 50°C, and N2 flowing at 30 ml/min served as the carrier gas. Ethylene was measured on a Perkin Elmer model 3920B gas chromatograph as described previously (2). Root systems were incubated for 10 min in 80-ml bottles before measuring H2. The bottles were then opened to air for 30 s before sealing and filling with 10% C2H2 in air (approximately 4 mM C2H2). Bottles were sampled for C2H4 after 3 and 8 min.

Uptake hydrogenase activity was measured by two methods. In both cases, excised lateral roots with attached nodules were placed in 24-ml assay bottles containing an atmosphere composed of 5% H2 + 10% C2H2 + 85% air. In all assays, a negative control consisting of unnodulated root tissue and a positive control consisting of Alaska/128C53 root nodules were included. In the first method, uptake hydrogenase activity was determined by the gas chromatographic disappearance of H2 during a 10-min period in the assay bottle. In the second, more sensitive method, 1H2 incorporation was measured after 30 min exposure to a normal assay atmosphere that had been supplemented to contain 0.167 μCi 1H2· ml−1 (3). Nodule samples were combusted in a Packard model B306 sample oxidizer, collected in Monophase 40 (Packard Instrument, Downers Grove, IL), and measured in a Beckman scintillation counter model LS8000. Free amino acid, soluble sugars, and starch content of root nodules were measured using colorimetric techniques reported elsewhere (15).

**RESULTS**

Root nodules of various pea cultivars grown in symbiosis with *R. leguminosarum* strain 128C53 evolved quite different amounts of H2 with similar rates of C2H2 reduction (Fig. 1). Initial experiments in this study had established that fact in several trials with the Alaska and J11205 lines before Dixon *et al.* (14) reported that strain 128C53 was Hup+ on Feltham First, the only pea cultivar in their study. Subsequent experiments in which Feltham First, Alaska, and J11205 in symbiosis with strain 128C53 were compared for H2 evolution and C2H2 reduction produced data com-
HOST EFFECTS ON RHIZOBIUM PHYSIOLOGY

Table I. Rhizobium Strain and Host Legume Cultivar Effects on Pea Root Nodule Activities at Flowering

Acetylene reduction, H2 evolution in air, and relative efficiency were measured under standard assay conditions. Hydrogen uptake was measured by GC. For those treatments in which no activity was detected, a maximum possible value of H2 uptake was calculated from the root nodule mass present and the sensitivity of the gas chromatograph. All values are the mean of six replicate plants.

<table>
<thead>
<tr>
<th>Pea Host</th>
<th>Rhizobium Strain</th>
<th>Acetylene Reduction</th>
<th>Hydrogen Evolution</th>
<th>Hydrogen Uptake</th>
<th>Relative Efficiency</th>
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<tbody>
<tr>
<td>J11205</td>
<td>128C53</td>
<td>530</td>
<td>8</td>
<td>150</td>
<td>0.98</td>
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<tr>
<td>J11205</td>
<td>3960</td>
<td>510</td>
<td>140</td>
<td>87</td>
<td>0.72</td>
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<tr>
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<td>300</td>
<td>520</td>
<td>370</td>
<td>&lt;1</td>
<td>0.29</td>
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<tr>
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<td>128C53</td>
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<td>47</td>
<td>180</td>
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<tr>
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<td>3960</td>
<td>360</td>
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<td>144</td>
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Host LSD (0.05) 54
Strain LSD (0.05) 101

Table II. Rhizobium Strain and Host Legume Cultivar Effects on Uptake Hydrogenase Activity in Pea Root Nodules at Flowering

Activities were determined with the H2 incorporation assay. Values can be multiplied by a factor of approximately seven to convert to the dry weight basis reported in Table I. In experiment II, a positive control consisting of root nodules from the Alaska/128C53 symbiosis had an uptake hydrogenase activity of 12.0 nmol H2-mg nodule fresh wt-1 h-1. Negative controls consisting of unnodulated root segments had apparent hydrogenase activities of 0.01-0.04 nmol H2-mg fresh wt-1 h-1 in both experiments.

<table>
<thead>
<tr>
<th>Pea Host</th>
<th>Rhizobium Strain</th>
<th>Hydrogen Uptake</th>
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<tbody>
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<td>1.5</td>
</tr>
<tr>
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<td>3960</td>
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<tr>
<td>Feltham First</td>
<td>300</td>
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Host LSD(0.05) 8.0
Strain LSD(0.05) 7.4

<table>
<thead>
<tr>
<th>Pea Host</th>
<th>Rhizobium Strain</th>
<th>Hydrogen Uptake</th>
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<tbody>
<tr>
<td>Feltham First</td>
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<tr>
<td>Feltham First</td>
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</tr>
<tr>
<td>Feltham First</td>
<td>300</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Strain LSD(0.05) 0.3

The plant cultivars measured to produce data in Figure 1 had quite different growth habits. Feltham First is a dwarf type which flowered 26 d after imbibition; Alaska had normal internode length and flowered after 26 d; J11205 had normal internode length, lacked tendrils, and flowered after 37 d. Plants that had the highest C2H2 reduction values for each cultivar in Figure 1 were assayed on days 36, 36, or 49 for Feltham First, Alaska, and J11205, respectively. On those dates six replicates of each of the three cultivars had the following values for total plant dry weight, nodule dry weight, and leaf area: Feltham First, 3.03 ± 0.17 g, 96 ± 9 mg, 279 ± 13 cm2; Alaska, 3.94 ± 0.06 g, 104 ± 6 mg, 328 ± 16 cm2; J11205, 4.04 ± 0.16 g, 134 ± 12 mg, 419 ± 37 cm2. Root nodules of those plants did not differ significantly (P ≤ 0.05) in analyses for free amino acids, soluble sugars, or starch. The mean values of the three cultivars for those parameters, respectively, were as follows: 22 μg leucine eq/mg nodule dry weight, 0.33 mg glucose eq/mg nodule dry weight, and 0.34 mg glucose eq/mg nodule dry weight.

The assay conditions employing 10% C2H2 (approximately 4 mM C2H2 under the normal temperature and pressure) did not permit any detectable H2 evolution either by the Feltham First/128C53 symbiosis (Fig. 2) or by Alaska and J11205 inoculated with strain 128C53 (data not shown). Further examination of symbioses involving strain 128C53 and the three pea cultivars at flowering showed that excess H2 evolution from Feltham First root nodules could be attributed to a lack of uptake hydrogenase activity in that cultivar (Table I). Those data, which were determined gas chromatographically by the disappearance of H2, showed similar effects of Feltham First on strain 3960. The host cultivar effects on C2H2 reduction and H2 evolution produced greater RE values for strains 128C53 and 3960 in J11205 and Alaska than in Feltham First. The Hup+ strain 300, however, had a nearly constant RE in all three cultivars, which was significantly less than the other two strains in all cases. The Hup+ phenotype of strain 128C53 in J11205 and Alaska and the
apparently Hup\textsuperscript{−} phenotype in Feltham First evident at flowering (Table I) also were observed at three other assay dates in these cultivars (data not shown).

Additional experiments using the sensitive \(^3\text{H}_2\) incorporation assay to measure uptake hydrogenase activity produced results that were qualitatively similar to those in Table I and showed that strains 128C53 and 3960 in Feltham First nodules expressed about 10\% of the hydrogenase activity they had in Alaska and J11205 (Table II). Statistical analyses of data in experiment I (Table II) showed a significant (\(P \leq 0.05\)) host effect of J11205 relative to Feltham First on both strains 128C53 and 3960. Strain 300 had significantly (\(P \leq 0.05\)) less uptake hydrogenase activity in both 128C53 and 3960 in Alaska and J11205 root nodules, but similar trends among the strains were not significant in Feltham First. Data obtained in experiment II were nearly identical to those in experiment I, but the design of the study allowed statistically significant differences among the strains to be detected in Feltham First (Table II). Thus, there was a significant (\(P \leq 0.05\)), detectable level of \(\text{H}_2\) uptake in strains 128C53 and 3960 on Feltham First, relative to strain 300.

The three pea cultivars used in these studies showed expected responses during the measurement of nodule activities on excised roots. Estimates of \(\text{C}_2\text{H}_2\) reduction, \(\text{H}_2\) evolution in air, and \(\text{H}_2\) uptake were linear over the time of the assays. The 5\% \(\text{H}_2\) concentration saturated uptake \(\text{H}_2\) activity in both assay techniques, and as reported previously (3), \(\text{C}_2\text{H}_2\) blocked \(\text{H}_2\) production by nitrogenase without preventing \(^3\text{H}_2\) incorporation.

**DISCUSSION**

Although previous work established that different host legume species can alter the uptake hydrogenase activity of a single *Rhizobium* strain (13, 20), the present study extends those findings to separate cultivars within *P. sativum*. Our data contrast with the apparent absence of any plant cultivar effect on hydrogen uptake by rhizobia associated with soybean and cowpea (7, 20). The very low level of uptake hydrogenase measured in *Rhizobium* strain 128C53 on Feltham First relative to Alaska and J11205 cultivars (Tables I and II) was reflected in much greater losses of \(\text{H}_2\) from Feltham First root nodules on a whole-plant basis (Fig. I). Calculations from Figure I show that while 36-d-old Feltham First and 37-d-old J11205 plants had identical whole-plant rates of \(\text{C}_2\text{H}_2\) reduction (25.0 \(\mu\text{mol} \cdot \text{plant}^{-1} \cdot \text{h}^{-1}\)), strain 128C53 evolved nearly 24-fold more \(\text{H}_2\) in Feltham First than in J11205 (11.35 \text{versus } 0.48 \mu\text{mol} \cdot \text{plant}^{-1} \cdot \text{h}^{-1}\). These data establish the validity of previous reports in the literature that strain 128C53 functions as a Hup\textsuperscript{−} phenotype on Alaska (3) and is Hup\textsuperscript{−} on Feltham First (14). The fact that Feltham First had a similar effect on uptake hydrogenase in strain 3960 relative to Alaska and J11205 cultivars (Table I) indicates that the phenomenon is not limited only to strain 128C53. Whether the Feltham First effect is specific for the symbiotic determinants on plasmid pRL6JI, which are present in both 128C53 and 3960, cannot be determined from the present study. It is possible that other *R. leguminosarum* strains carry Hup\textsuperscript{−} determinants that are expressed in Feltham First.

Host cultivar effects on uptake hydrogenase activity may not be solely responsible for the differences in \(\text{H}_2\) evolution reported in Figure I. It is conceivable that EAC, and thus \(\text{H}_2\) production by nitrogenase, also was affected by host plant genotype, but this suggestion is not supported by the data indicating that RE was similar for the Hup\textsuperscript{−} strain 300 in J11205, Alaska, and Feltham First at flowering (Table I). In such a Hup\textsuperscript{−} strain, the RE should provide an indication of the apparent EAC because nitrogenase would be the only enzyme system affecting \(\text{H}_2\) evolution. It is not possible (\(P \leq 0.05\)) to dismiss the possibility of the results simply by examining RE in strain 128C53 or 3960 because both organisms had uptake hydrogenase activity on J11205 and Alaska. One reason for considering such a hypothesis is that recent data show the apparent EAC of the Hup\textsuperscript{−} *R. leguminosarum* strain 3740, which is closely related genetically to strains 300 and 3960 (4, 9), was altered by environmental treatments of the host Alaska pea (15). Although the results in that study were tentatively ascribed to changes in soluble sugar concentration in the root nodules, which did not differ significantly among the three cultivars in the present experiments, it is possible that other cultivar-specific compounds could be altered.

The mechanism by which Feltham First produced a Hup\textsuperscript{−} phenotype in *Rhizobium* strains 128C53 and 3960 relative to Alaska and J11205 cannot be determined from the results in this study. It is possible that shoot factors translocated to the root nodule, including photosynthesize generally or more specific compounds, affected uptake hydrogenase activity. It is equally reasonable to postulate that leghemoglobin altered the availability of \(\alpha_8\), which serves as a terminal electron acceptor for the uptake hydrogen system. Those possible differences between Feltham First and the other pea materials, however, cannot be examined meaningfully by comparing such genetically and physiologically diverse cultivars. grafting experiments involving different cultivars may separate shoot and root effects, but mechanisms operating at the molecular level probably will require comparisons between plants that are genetically more similar than those in this study. It is possible that the uptake hydrogenase protein was present in the 128C53 bacteroids in Feltham First root nodules, but some plant-dependent physiological mechanism decreased its activity. Such a possibility is not supported by bacteroid studies reported by Dixon (13) in which cells of *R. leguminosarum* ONA 311 maintained a Hup\textsuperscript{−} phenotype after isolation of *Vicia faba* root nodules while showing a Hup\textsuperscript{−} phenotype after isolation from *P. sativum* root nodules. An alternative hypothesis that Feltham First failed to induce synthesis of uptake hydrogenase is consistent with the low but detectable levels of hydrogenase activity measured for strains 128C53 and 3960 in that cultivar (Table II, experiment II). Such low levels of enzyme activity, amounting to about 10\% of the fully induced condition, have been reported in bacterial cells with mutations in a promoter region of glutamine synthetase (21). Genetic mechanisms operating on related proteins, such as electron carriers associated with uptake hydrogenase (17), also could explain the results observed in this study.

The biological significance of observations in this study are unclear. Data from isolated soybean bacteroids indicate that uptake hydrogenase activity can promote \(\text{C}_2\text{H}_2\) reduction and increase the steady-state pool of ATP under optimum \(\text{H}_2\) concentrations (18). Results from other experiments suggest that uptake hydrogenase or some associated genetic trait improves symbiotic performance of *R. japonicum* (1). It is conceivable, therefore, that a pea plant could benefit from the capacity to facilitate uptake hydrogenase activity in *R. leguminosarum*. The very great differences in growth and development among the three pea cultivars in this study, however, preclude studies on the effects of variation in \(\text{H}_2\) evolution on those parameters. If one were successful in developing isogenic lines of peas with high and low \(\text{H}_2\) evolution from the same *Rhizobium* strain, then it would be possible to test whether host plant control of uptake hydrogenase in the symbiotic *Rhizobium* confers any significant advantage to the plant. The presence of the recessive alleles controlling dwarfism and lack of tendrils in Feltham First and J11205, respectively, is abetting that project.

The observations in the present study may have significant agronomic implications. If it can be established that host plants producing Hup\textsuperscript{−} traits in *Rhizobium* have advantages over those carrying Hup\textsuperscript{+} phenotypes, then the generality of the phenomenon should be considered seriously. Specifically, the absence of Hup\textsuperscript{+} phenotypes of *Rhizobium* in alfalfa and clover should be examined in this light. Of 19 strains of *R. meliloti* tested on one cultivar of alfalfa, all were incapable of oxidizing significant amounts of \(\text{H}_2\).
However, two of those strains showed low levels of uptake hydrogenase in pure culture under conditions known to derepress the Hup\(^+\) phenotype in \textit{R. japonicum}. Thus, it can be suggested from results in the present report that the Hup\(^+\) phenotype in those two \textit{R. meliloti} strains was suppressed or not activated in the single alfalfa cultivar used for the trials. In a similar manner it can be suggested that although no strains of \textit{R. trifolii} tested on a single cultivar of white clover were Hup\(^+\) (27), the same strains might show a different phenotype on other cultivars. This latter possibility is more hypothetical than the case in \textit{R. meliloti} because it is not known whether any of the \textit{R. trifolii} strains tested had genetic determinants for uptake hydrogenase. Nevertheless, these facts suggest that the results reported here for pea may be applicable to the agronomically important forage crops alfalfa and clover.

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