STIMULATED NODULATION OF SOYBEANS BY RHIZOBIUM JAPONICUM MUTANT (B-14075) THAT CATABOLIZES THE CONVERSION OF TRYPTOPHAN TO INDOL-3YL-ACETIC ACID*

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Nodulations of Glycine max L. seedlings by parental Rhizobium japonicum (USDA strain 26) and a tryptophan catabolic mutant (tan 4b, NRRL strain B-14075) were evaluated for effectiveness. Seedlings grown in plastic pouches were compared based on increases in root weight (wt.), nodule volume (vol.) and acetylene-reducing (AR) activity. The tan 4b mutant, which can produce extracellular indol-3yl-acetic (IAA) and indol-3yl-pyruvic (IPA) acids asymbiotically, enhanced nodulation significantly. Nodulation by strain 26 was most effective when a basal nutrient was supplemented with sucrose, CaCO₃, EDTA, nicotinic acid and glutamate. In contrast, effective nodulation by tan 4b did not require an exogenous glutamate (0.34 mM)-nicotinate (81 μM) combination. Like strain 26 inoculum supplemented with exogenous IAA (0.1 mM), taproot formation associated with unsupplemented tan 4b was inhibited by exposure to direct light.

Key words: Glycine max-Rhizobium symbiosis; tryptophan-catabolic mutants (tan); nodulation assay

Introduction

IAA has been shown to stimulate root growth [1,2] and to affect other organs of leguminous plants [3]; but its role in nodulation is unresolved [4]. IAA formed in apical shoots and leaves is translocated basipetally to roots through vascular tissues of the stem. In this connection, it is of interest that soybean roots either treated with IAA or inoculated with IAA-producing Rhizobium synthesized a specific polypeptide, which is thought to be involved in development of root nodule symbiosis [5].

Recently, tryptophan catabolic mutants (B-14075, B-14076), designated tan and derived from R. japonicum USDA 26, were characterized by their capacity to convert exogenous tryptophan to extracellular IAA and IPA [6]. Other rhizobia have been found to produce IAA without affecting legume nodulations [4,7]. The tan mutants are unusual in that they produce up to 30-fold more indolyl than parent strain 26; strain 26 forms no detectable IPA from exogenous tryptophan. Tan mutants also retain capabilities of the parental strain to reduce acetylene asymbiotically [6] and to nodulate soybeans effectively [8]. These characteristics suggested that, when tryptophan catabolic mutants are used as inoculants, symbiotic nitrogen-fixation might be enhanced by endogenous, auxinic stimulations of the...
infected soybean roots. Such experiments, conducted in plastic growth pouches with various exogenous nutrients, demonstrated that effective nodulations by tan 4b were indeed enhanced with respect to root fresh wt., nodule vol. and AR activity.

Materials and methods

A plastic growth pouch method [9-11] was modified as a means to determine stimulatory effects on both root growth and nodulation. Beforehand, a number of procedural steps were examined to ascertain statistical reliability of the method. Both fresh root wts. and effective nodulations were used to identify and compare efficient symbioses.

Soybean growth in plastic pouches

Seeds of G. max L. cv Clark L-1 were surface-sterilized in ethanol for 2 min, treated with 1% (w/v) sodium hypochlorite for 10 min, and washed thoroughly with sterilized water. The soybeans were then suspended aseptically in water, aerated overnight at 27°C with a sparger, and sterilized with hypochlorite a second time. By using this aseptic procedure, all 17 pouches from 4 sets of uninoculated soybeans were nodule-free. If uninoculated plants did not nodulate, examination of 2 × 2 contingency tables [12] indicated that significant difference is attained with an exact probability above 95% using a minimum of 4 pouches/treatment. Accordingly, three swollen, germinating seeds were inoculated [13] with specific Rhizobium and planted in each plastic pouch (15 × 15 cm; from Northrup, King & Co., Minneapolis, MN) containing 25 ml plant nutrients. Each treatment or set of seedlings was replicated in 5-10 sterilized pouches and incubated aseptically under the folded flaps of the plastic pouches. All soybean seedlings were cultivated on shelves illuminated by a mixture of warm-white fluorescent, incandescent filament, and natural lighting (16 h light, 5-15 Wm⁻²) at ambient temperatures until emergence of epicotyls (5-7 days).

The 7-day plants were thinned to 1 plant/pouch and the troughs of pouches were covered with sterile, non-absorbent cotton. Plants were transferred to a growth chamber (16 h light, 25°C) irradiating 25 Wm⁻². Each pouch received three additions (5 ml, 3 ml, 3 ml) of nitrogen (N)-free basal nutrients [13] at weekly intervals. Water that evaporated from growth pouches was replenished to the 20-40 ml levels. Plants of the same set were harvested on the same day (20-30 days after inoculation) for comparisons. Under similar growth conditions, early nodulations in either plastic pouches or vermiculite-sand soils were detectable about 2 weeks after inoculation.

Analysis of efficient symbiosis

Each plant was cut at the stem base, and the intact root system was weighed. Effective symbiotic nodulation/root was determined in two ways: by estimated relative vol. of all nodules (kd³ where k is π/6 and d is diameter in mm) and by AR activity (nmol ethylene produced h⁻¹). Relative nodule vol./root was estimated by counting the sum of nodules and grading them into three groups: large nodule with approx. 4 mm diameter (8 k mm³ vol), medium with 2 mm (1 vol.), and small with <1 mm (0.13 vol.). AR activity h⁻¹ was measured by gas chromatography after the stemless roots with intact nodules were sealed in 25-ml vials with an atmospheric mixture of 4 kPa acetylene and 100 kPa air.

Mean values, standard errors of the mean (S.E. = s/√n), and F-ratio tests were calculated. Analyses of variance (comparative F-ratios where P < 0.05), similar to that outlined by Brockwell [14], were used to establish significant treatment differences. Least significant difference values (LSD at the P < 0.05 level) were calculated where the extent of difference in effective nodulation was estimated.

R. japonicum USDA 26, synonymous to NRRL strain L-259, and its derived mutant tan 4b, synonymous to B-14075, were compared as symbionts under various plant
growth conditions. Specific nodulations by tan 4b were identified by their tan- to orange-colored (IPA producing) colonies, when surface-sterilized nodules were crushed and streaked onto agar media containing 300 mg L-tryptophan/l. Each inoculum contained approximately \(10^7\) colony-forming units/ml when plated out on a yeast extract-mannitol-glucanate-soil extract agar medium. A prior experiment and \(F\)-test \((P<0.05)\) indicated that other slow-growing rhizobia had significantly different capacity to nodulate cv. Clark L-1 roots \([8]\); the AR activity varied from 220 to 440 nmol h\(^{-1}\) g wet wt\(^{-1}\).

**Nutrient media for root growth**

In addition to rhizobial inoculum, root growth in plastic pouches was found to increase because of exogenous nutrient supplements. To identify some of these stimulatory factors, a N-free mineral medium described by Wacek and Brill \([13]\) and modified to contain 4 \(\mu\)M nickel supplement \([15,16]\) was used as a basal growth medium. All pouches also contained exogenous supplements of 23 mM sucrose and 20 mM insoluble CaCO\(_3\) \([11]\). Supplements (5 pouches/treatment) were added only once into sterilized pouches containing an initial 25 ml of plant nutrients. Exogenous N-supplements of 0.34 mM L-glutamate, 1 mM NH\(_4\)Cl, and 1 mM KNO\(_3\) were compared in terms of root growth and nodulation with symbiont strain 26. Other exogenous supplements consisting of high levels (74 mM) of EDTA and of nicotinic acid cofactor (81 \(\mu\)M), reported to stimulate nodulation \([17]\) and root growth \([18]\) respectively, were also evaluated.

**Effect of exogenous IAA**

Because IAA was added only at the beginning of a 3.5-week growth period, a large excess (0.11 mM) was used in order to elicit measurable growth responses of nodules and root tissues as well. IAA in combination with exogenous L-glutamate (0.34 mM) and nicotinate (81 \(\mu\)M) was added into growth pouches; and the germinating seedlings were inoculated with either strain 26 or tan 4b mutant. Root stimulation elicited by either the nutrient factors or different symbionts (5 replicate pouches/treatment) was determined by root fresh wt. and nodulations. To evaluate exogenous IAA, it was necessary to grow pouched plants without exposing the root systems to direct light. When exposed to light, the IAA-treated taproots emerging from the unexposed areas of paper troughs became necrotic and black; but delayed secondary roots eventually emerged in place of the inhibited taproot.

**Results and discussion**

The reliability and limitations of the growth pouch method were explored in preliminary experiments. For example, plants were thinned to 1/pouch because measurement of root wt. (12 pouches treated the same way) disclosed interpretive problems dependent on the number of plants/pouch. In addition, AR activity of nodulated roots was shown previously to depend on specific rhizobial inoculant \([8]\). Consequently, the growth pouch method can be used to evaluate effective nodulations only when plants/pouch are held constant.

Preliminary factorial experiments with strain 26 tested in as many as 2\(^4\) combinations confirmed other investigations that a 3-way combination of (i) sucrose supplements in 8 g/l concentration \([13]\), (ii) 50 mg CaCO\(_3\) additions into troughs \([11]\), and (iii) direct light on roots increased nodulated roots significantly \((F\)-test where \(P<0.1\) or \(<0.05)\) over ineffective combinations. Like sucrose (23 mM), trehalose (23 mM) and glucose (12 mM) stimulated effective nodulations (approx. 1.1 g root wt. with 360 nmol ethylene h\(^{-1}\) g\(^{-1}\) AR activity). Such carbon supplements (5 pouches/treatment) as gum arabic, soluble starch, lactose, maltose, fructose, inositol, mannitol-glucanate combination and succinate were either less effective or ineffective when compared with sucrose. Other growth factors such as kinetin (0.4--4 mg/l), gibberellic acid...
(0.4—2 mg/l), adenine sulfate (10 mg/l), and trigonelline (2 mg/l) also elicited less effective nodulations.

Nodulations by strain 26 (26 pouches with 4 different treatments) displayed significant correlation of AR activity with root wt. \((r = 0.64)\) and estimated nodule vol. \((r = 0.74)\). In contrast, pH measurements of residual nutrients at harvest time, which dropped from an initial pH 6.5 to harvest-time pH 4, gave no correlation with AR activity \((r = -0.09)\). Thus, effective symbioses are usually but not always associated with good root formations.

**Root symbiosis stimulated by exogenous glutamate, EDTA, and nicotinate nutrients**

In confined plastic pouches, soybean plants and their roots were readily restricted by growth conditions. Specifically, exogenous nutrients (Table I) as well as such growth conditions as specific symbionts, temperature, nutrient pH, and light could be manipulated to study their effects on the symbioses. With *R. japonicum* strain 26, nodulated roots grew better with a combined supplement of 0.34 mM glutamate, 74 μM EDTA, and 81 μM nicotinate than in either unsupplemented or partially supplemented basal nutrients (Table I). Root wt. (1.6 g) and AR activity (800 nmol h\(^{-1}\) g\(^{-1}\)) were higher when the combined supplements were added than when roots formed in an unsupplemented medium. These increases of root wt. \((P < 0.01\) level) and AR activity \((P < 0.03\) level) were significant when evaluated by F-tests. Moreover, the comparable S.E. of AR activities (approx. ±70) suggests that these analyses of the nodulated roots were reproducible at similar levels whether or not roots were grown with combined supplements.

**Root nodulation in response to exogenous IAA and tan inoculum**

Systemically formed IAA can elicit a hormonal response by altering plasma membrane of vascular-bundle sheaths [2]. In cut stems, new root tissues induced by IAA pretreatment are formed when sugars are translocated basipetally to the stem base [1]. It follows that endogenous IAA can facilitate and, thereby, coordinate the translocation of nutrients to growing roots. In contrast to systemically formed IAA, high concentrations of exogenous IAA (10—40 mg/l) were toxic to germinating seedlings and did not stimulate root growth even when the roots were protected from direct illumination. Of 20 such pouched plants (Table II) inoculated with

**Table I.** Root growth and nodulation of soybeans cv. Clark L-1 in plastic growth pouches dependent on exogenous nutrient supplements. All seedlings were inoculated with *R. japonium* (USDA 26) and grown in a basal nutrient medium supplemented with 23 μM sucrose and 0.34 mM CaCO\(_3\). Planta were harvested approx. 3.5 weeks after inoculation. Data are mean values for root systems from composite sets (see Materials and methods).

<table>
<thead>
<tr>
<th>Exogenous nutrient supplements</th>
<th>Root growth/plant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of plants</td>
</tr>
<tr>
<td>None</td>
<td>21</td>
</tr>
<tr>
<td>Glu(^a) (0.34 mM)</td>
<td>9</td>
</tr>
<tr>
<td>EDTA (74 μM)</td>
<td>10</td>
</tr>
<tr>
<td>Combined Glu, EDTA and nicotinate(^b) (81 μM)</td>
<td>6</td>
</tr>
</tbody>
</table>

\(^a\)NH\(_4\)Cl (1 mM) and KNO\(_3\) (1 mM) did not replace L-glutamate (Glu) for symbiotic root stimulations (190 and 250 nmol h\(^{-1}\) g\(^{-1}\) AR activity, respectively).

\(^b\)In combined nutrient supplements, root systems did not require exposure to direct light for stimulation.
Table II. Comparative nodulations associated with *R. japonicum* USDA 26 and tan 4b. Plants were harvested 3.5–4 weeks after inoculations. Nutrient medium and analyses as in Table I.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Exogenous nutrient supplements</th>
<th>Root growth/plant</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of plants</td>
<td>Fresh wt. (g)</td>
<td>Estimated nodule-vol. (kmm³)</td>
</tr>
<tr>
<td>USDA 26</td>
<td>None</td>
<td>4</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>Glu (0.34 mM) – nicotinate (81 μM)</td>
<td>8</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>Glu-nicotinate and IAA (0.05 to 0.22 mM)</td>
<td>20</td>
<td>0.36</td>
</tr>
<tr>
<td>Tan 4b</td>
<td>None</td>
<td>5</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>Glu-nicotinate</td>
<td>12</td>
<td>1.51</td>
</tr>
<tr>
<td></td>
<td>Glu-nicotinate and IAA (0.11 mM)</td>
<td>6</td>
<td>0.52</td>
</tr>
</tbody>
</table>

*Mean of 7 plants that were nodulated.*

*Mean of 3 plants that were nodulated.*

Strain 26, only 7 formed root nodules. These had variable AR activity (ranged from 50 to 920 nmol h⁻¹ g⁻¹). Furthermore, most of the surviving plants were stunted (mean of 0.36 g root wt.). The taproots were completely inhibited if developing root systems were exposed to light; and secondary roots grew in place of the light-inhibited taproot. Exogenous IAA (0.1 mM) was also toxic when combined with tan 4b inocula.

Because tan mutants produce IAA and IPA from tryptophan [6], it seemed probable that these mutants would elicit an auxin-like response if integrated into root tissues. Unlike strain 26, tan mutant 4b stimulated significant soybean root growth (1.3 g/plant) and AR activity without use of the glutamate-nicotinate nutrient combination (Table II). Even though the combined supplements increased fresh root wt. slightly (1.5 g/plant), seedlings nodulated with tan 4b yielded relatively high nodule vol. (37 kmm³) and AR activities (approx. 1000 nmol h⁻¹ g⁻¹) regardless of glutamate-nicotinate supplementations. However, AR activity by roots nodulated with strain 26 was significantly (*P < 0.01*) increased by glutamate-nicotinate supplementations to approx. 900 nmol h⁻¹ g⁻¹ when compared to unsupplemented plants.

As with strain 26 supplemented with exogenous IAA, infection with unsupplemented tan 4b also led to inhibition of taproot formation (1.13 g root wt., 18 kmm³ relative nodule vol., and 660 ± 130 nmol h⁻¹ g⁻¹ AR activity) when the roots were directly illuminated. Clusters of nodules were formed within the covered troughs along unexposed portions of the taproots. The results therefore suggest that tan 4b produces auxinic substances within infected roots and replaces the glutamate-nicotinate supplement. Whether tan mutants can similarly enhance nodulation of pot- or field-grown plants would indeed be of interest.

References


