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A method for screening *Phaseolus vulgaris* L. germplasm for preferential nodulation with a selected *Rhizobium etli* strain

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Abstract

As part of a breeding program to improve the nitrogen-fixing symbiosis between common bean (*Phaseolus vulgaris*) and *Rhizobium etli*, we developed a rapid screen for common bean accessions that preferentially nodulate with KIM5s, a high nitrogen fixing strain of *R. etli*. We constructed a mutant of KIM5s that did not fix nitrogen (Fix$^-$) but was otherwise indistinguishable from KIM5s. We screened plants for symptoms of nitrogen deficiency when grown in a Honduran soil containing indigenous common bean-nodulating rhizobia (10^4 per gram) and KM6001, the Fix$^-$ mutant of KIM5s (10^4/seedling added 7 days after planting). Leaf color was scored on a scale of 1 to 5, in which 1 was dark green and 5 was bright yellow. Of 820 genetically diverse accessions of *P. vulgaris* screened, 51 were scored 1, 626 were scored 2 or 3, and 143 were scored 4 or 5. Selfed seed was produced from common bean plants of the accessions scored 1, 4 or 5. Twenty-four accessions that scored 1, and 58 that scored 4 or 5 were screened in soil containing indigenous rhizobia and the wild type KIM5s (Fix$^+$), and nodule occupancy was determined by antibiotic resistance. On the 24 common bean accessions that were scored 1, KIM5s occupied 0–6% of the nodules, on 26 of the accessions that were scored 4 or 5, KIM5s occupied 90%–100% of the nodules, and on the remaining 34 that scored 4 or 5, there was a distribution of nodule occupancy. Foliar color was highly correlated with nodule occupancy (r = 0.786, p = 0.01). The results indicate that the rapid visual screen using the Fix$^-$ mutant accurately identified common bean accessions that preferentially nodulate with the wild-type KIM5s (Fix$^+$) strain in soil containing indigenous rhizobia. This screen will facilitate introduction of the preferential nodulation trait into superior cultivars and provides the foundation for studies of the genetic basis of preferential nodulation.

Introduction

Nitrogen availability is often a problem for production of common beans, particularly in developing countries where nitrogen fertilizers are either unavailable or unaffordable (Graham, 1981). Despite their unusual ability to enter into intimate association with nitrogen-fixing symbionts, productivity of *Phaseolus vulgaris* L. is often limited by nitrogen deficiency under agronomic conditions. One of the reasons for this apparent paradox is that many of the indigenous strains of *Rhizobium etli* bv.*phaseoli*, which nodulates common beans, are poor in nitrogen fixation (Dowling and Broughton, 1986; Moxley et al.,1986). Although high nitrogen fixing strains of *R. etli* have been identified, they often do not provide an agronomic benefit in the field because they are excluded from the nodules of the host plant by the indigenous *Rhizobium* strains in the soil, which are often more competitive for nodulation than strains applied as inoculants (Graham, 1981). The problem of nodulation competitiveness has been studied for over 50 years (Maier and Triplett, 1996; Streeter, 1994), but little progress has been made in understanding the biological basis for competitiveness or in overcoming the problem for agronomic gains. Achieving nodule occupancy by desirable strains of

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Rhizobium remains the most significant barrier to extracting practical benefit from nitrogen fixation for agriculture.

Strategies to overcome the competitiveness of the indigenous *Rhizobium* strains and achieve nodule occupancy with inoculum strains have largely focused on the bacteria. Extremely high inoculum levels (Weaver and Frederick, 1974), pretreatment of the bacteria with root exudate from the host (Bhagwat and Thomas, 1993), formulation, placement, or inoculum carriers (Paau, 1991; Roughley et al., 1993; Streeter, 1994), and strains improved by mutation and genetic engineering (Maier and Triplett, 1996; Paau, 1991), and bacteriocin production (Triplett, 1990) have been shown to improve nodule occupancy by the desired strain. Little attention has been paid to the role of the host genotype in achieving nodule occupancy by desirable strains, although plant breeding has proven to be a powerful approach to improving the ability of the host to support nitrogen fixation (Bliss, 1985).

A few studies indicate that breeding for preferential nodulation has potential. Kipe-Nolt et al. (1992) found a tendency for common bean genotypes of Middle American origin to prefer CIAT632, a *R. etli* strain isolated from Guatemala, and that Andean accessions form effective nodules more quickly with CIAT899, a broad host-range, *R. tropici* from Colombia. In that study the strains were applied individually and thus, competition was not studied. Soybeans have been bred successfully to exclude the dominant strains of serogroup 110 of *Bradyrhizobium japonicum* in Midwestern U.S. soils. Cregan et al. (1989) identified accessions of soybeans that would not nodulate with the undesirable strains and then introduced this trait into other genetic backgrounds. The inability to nodulate with serogroup 110 is a simply inherited, dominant trait, thus resembling many genes for disease resistance (Lohrke et al., 1996).

We propose the converse approach to the one used for soybeans – to breed the host plant for preferential nodulation with a desirable, high nitrogen fixing strain. We chose common beans as the system because the nodulation competition problem is significant for the host, the plant is important to human nutrition, interest in common bean genetics has increased since the recent identification of new sources of useful germplasm and the development of gene mapping techniques (Nodari et al., 1993), and the symbiont of common beans, *R. etli*, can be manipulated genetically (Bittinger et al., 1997; Noel et al., 1983; Triplett, 1990). To develop an efficient breeding program for preferential nodulation in common beans, we needed a rapid screen for nodule occupancy to identify germplasm and to follow the preferential nodulation trait in segregating populations. Here we describe a rapid, visual screen for plant lines that preferentially nodulate with a desirable strain of *R. etli* using a mutant strain that does not fix nitrogen.

Materials and methods

Plant material

A diverse common bean (*Phaseolus vulgaris* L.)germplasm collection of 820 accessions was utilized in this study. This collection included accessions from all of the dry common bean market classes suggested by Voysest (1983) obtained from the research collections maintained at the Escuela Agricola Panamericana (EAP), Zamorano, in Honduras, from J. Beaver, University of Puerto Rico, K. Kmiecek, University of Wisconsin, J. Kipe-Nolt, formerly at CIAT, Cali, Colombia, and R. Henson, formerly with the Bean/Cowpea CRSP-Ecuador project. Seventy-five accessions, mostly landraces collected in Honduras during 1990–92 were also included. For the studies reported here, the accessions were increased in the greenhouse at Zamorano.

Growth medium

A plant growth medium containing soil from Zamorano was developed and guided by two requirements: the presence of a sufficiently high indigenous *Rhizobium etli* bv. *phaseoli* population to provide competition to the KIM5s Fix$^-$ mutant strain that was used as inoculum, and a low nitrogen (N) content to facilitate the visual identification of N-deficiency symptoms in plants nodulated by the Fix$^-$ strain. The optimum medium was found to be a mixture of soil, perlite, vermiculite and sand (1:1:1:1), which had approximately 0.02% of N and 10^4 *R. etli*/g medium, as determined by MPN (Vincent, 1970). The soil for preparing the growth medium came from the plot identified as Terraza 6 Agronomia at Zamorano, Honduras. Once prepared, the medium was supplemented with 0.05 g of 0–46–0 fertilizer and 1 g of $CaCO_3$ per kg of medium.

Fix$^-$ strain

We constructed a Fix$^-$ mutant that is nearly isogenic to KIM5s by marker exchange mutagenesis. A Tn*5* insertion was cloned from a Fix$^-$ mutant, CE108, of strain CE3 (Noel et al., 1984), and the clone was introduced into KIM5s on pSUP202, a plasmid that does not replicate in KIM5s. The insertion was selected with kanamycin (Km) at 200 μg/mL. Loss of the plasmid was determined by sensitivity to chloramphenicol (Cm) at 10 μg/mL. Most of the mutants selected by the method were resistant to both km and cm, indicating that the plasmid had inserted in the genome by a single recombination event. Approximately 1% of the km^r mutants were cm^s, indicating a double recombination event; these mutants were studied further. The mutants were tested on plants for nodulation and acetylene reduction activity. Plants were scored for leaf color 3 weeks after inoculation with the mutants. We identified five mutants that nodulated normally, induced symptoms of N deficiency, did not reduce acetylene, and had an insertion that was indistinguishable from the insertion in CE108 as shown by Southern blot analysis (data not shown). One mutant, designated KM6001, was used as inoculum for the screening of germplasm for preferential nodulation.

Screening germplasm for preferential nodulation

The 820 accessions from the diverse common bean germplasm collection were screened in the greenhouse for preferential nodulation using mutant KM6001. Accessions were subdivided in 15 groups of 56 genotypes. Six control treatments were cultivars Puebla 152 and Sanilac planted in sterilized soil medium, inoculated with the Fix$^-$ strain and grown with or without added N fertilizer. The experimental design was a complete randomized block with five replicates. Six seeds per accession were planted in 15-cm (dia.) standard plastic pots containing 1.12 kg of soil medium. At the V2 stage of development (primary leaves), approximately 7 days after planting (DAP), all except two plants per pot were cut off at the soil with scissors. The remaining plants were each immediately inoculated with 1 ml of culture of the KM6001, the Fix$^-$ mutant. The inoculum contained 10^4 bacteria/mL, which is similar to the indigenous population present in the growth medium. Plants were generally watered with tap water twice daily, alternating with an afternoon irrigation every two days with the N-free solution of Broughton and Dilworth (1970). Plants under N treatments were irrigated similarly with the addition of 70 ppm of N in the form of KNO_3.

The rating of the common bean germplasm for foliar coloration was conducted on five plants of each accession 21 days after inoculation (DAI), which was 28 DAP, at approximately the V4 stage of development (first trifoliolate leaf), using a 1 to 5 scale (1 = dark green, 2 = light green, 3 = yellowish green, 4 = light yellow and 5 = bright yellow). Nodulation was assessed by removing the plants carefully and then rating them with the standard system for common bean germplasm evaluation from CIAT, which is a qualitative scale based on evaluation of nodule number and size of pink nodules.

Plants with scores of 4 or 5 were immediately transplanted to pots containing a soil medium with optimum levels of N, where they grew to maturity, at which time seeds were harvested for nodule occupancy studies. Similarly, nodulated plants that were scored 1 (dark green leaf color), indicating no N-deficiency symptoms and therefore suggesting that nodulation was mostly due to indigenous rhizobia present in the soil medium, were also transplanted and grown to maturity. Plants with intermediate scores of 2 and 3 were not transplanted at this time; however, these accessions were increased using seed from the original lots.

Determination of nodule occupancy

Genotypes were tested for preferential nodulation with the wild type strain KIM5s (Fix$^+$). Nodule occupancy by KIM5s was determined in accessions that were scored 1, 4 or 5. Plants were grown as described above with the following modifications. The inoculum strain used for nodule occupancy determination was KIM5s, which is spectinomycin resistant. The experimental unit was a single plant per pot, and the experimental design was a complete randomized block with 10 replicates.

Nodules were removed 21 DAI. Plant shoots were removed with shears and the roots were removed carefully from the pots and rinsed with tap water. Then 8–10 nodules from the primary root were removed with forceps, placed in vials with 1.5 mL of 15% glycerol, and stored at -4 °C. In the laboratory, five nodules from each vial were immersed in 95% ethanol for 10 s, then placed in 2% sodium hypochlorite for 1 min for surface sterilization, and rinsed in sterile distilled water. The nodules were placed individually in wells of a sterile round-bottomed polystyrene, 96-well microtiter plate containing 120 μL of YM broth (Wacek and Brill, 1976) per well. Nodules were crushed with a multiple crusher/inoculator apparatus, flame-sterilized with ethanol, positioned in the wells

Table 1. Visual assessment of leaf color of 820 common bean accessions inoculated with KM6001

Market class	Visual score[a] 1	2	3	4	5	Total
Small black	9	49	61	15	1	135
Small red	13	100	101	36	1	251
Pink	2	16	13	4	0	35
Red mottled	1	22	71	37	2	133
Cream mottled	9	9	12	2	0	32
White	1	26	36	11	1	75
Cream	4	24	13	7	1	49
Light yellow	0	5	8	6	0	19
Dark yellow	2	3	4	1	0	10
Grey	1	2	0	0	0	3
Dark grey	1	1	1	0	0	3
Brown	3	5	1	1	0	10
Striped	3	20	18	17	0	58
Mixture	2	2	3	0	0	7
Total	51	284	342	137	6	820

[a] Foliar coloration rating scale: 1 = dark green; 2 = light green; 3 = yellowish green; 4 = light yellow; 5 = bright yellow; plants were evaluated at 21 DAI (28 DAP). Values represent the mean of ten plants (five pots of two plants each).

of the microtiter plate, and gently pushed to crush the nodules (Beattie and Handelsman, 1989). The apparatus was then dipped into the wells and 48 drops of bacterial suspensions were carried on the bolts and transferred onto a YM plate containing Congo red (Bastarrachea et al., 1988). Repeated transfers were then made to YM plates containing spectinomycin, the indicator antibiotic. The plates were placed in an incubator at 30 °C for 3 days for evaluation of growth. Positive bacterial growth in plates containing spectinomycin was the development of 10 or more colonies in each drop.

Results

The prediction underlying the development of this rapid screen was that if a Fix$^-$ strain occupies most of the nodules of plants grown under low N, then the plants will turn yellow due to N deficiency. Therefore, use of a Fix$^-$ mutant of KIM5s could provide a visual screen for host genotypes that are preferentially nodulated by KIM5s, and the phenotype could then be verified by direct assessment of nodule occupancy.

Screen for preferential nodulation

The results from the visual screening of 820 accessions inoculated with KM6001, the Fix$^-$ strain, indicated that most common bean market classes were represented in each phenotypic group as indicated by the foliar assessment. Exceptions were the market classes with a small number of accessions included in the screening. The phenotypic classes are represented among all of the market classes (Table 1). Accessions that were scored 1 were those showing dark green leaf color, i.e. no symptoms of N-deficiency. Accessions that were scored 4 or 5, were those showing light or bright yellow leaf color, an indication of N-deficiency. A total of 51 accessions (6.2%) were scored 1, 284 (34.6%) were scored 2, 342 (41.7%) were scored 3, 137 (16.7%) were scored 4, and 6 (0.7%) were scored 5. Highly significant differences in foliar coloration ($P < 0.001$) were found among accessions. All of the accessions that turned yellow appeared to nodulate normally except for two, which are the poor nodulating and low N_2-fixing navy bean line Sanilac and the non-nodulating line NOD 125.

Nodule occupancy

To determine whether foliar appearance provided an accurate indicator of nodule occupancy, we chose 24 accessions that score 1 and 58 accessions that scored 4 or 5 when inoculated with KM6001 in the presence of indigenous rhizobia, and tested them for nodule occupancy by KIM5s (Fix$^+$). The 24 accessions scored 1 were nodulated minimally or not at all by KIM5s (0% to 6% nodule occupancy), whereas 26 accessions from a total of 58 (44.8%) that scored 4 or 5 had a high frequency of nodules containing KIM5s ($\geq$90%) (Table 2). There was a significant correlation between foliar color scores and nodule occupancy values (r = 0.786; $P < 0.01$) from the 82 accessions tested for nodule occupancy. These data indicate that the foliar assessment of plants inoculated with the Fix$^-$ mutant strain is a good predictor of nodule occupancy by KIM5s (Fix$^+$).

The distribution of the common bean accessions tested for nodule occupancy with KIM5s strain shows that all of the 24 accessions scored 1 fall within the 0–10% nodule occupancy class (Table 3); and that a relatively high number of accessions (62.1%) that were scored 4 or 5, fall within the three upper most classes (nodule occupancy 71–100%) (Table 3). Lines that were exceptions to the association between nodule occupancy and foliar color were 9 of the 11 accessions from the lowest nodule occupancy group (nodule

Table 2. Nodulation characteristics of common bean accessions with low or high leaf color scores

Accession	Market class[a]	Nodulation[b]	Visual score	KIM5s nodule occupancy (%)[c]
S-182-V	Small black (M)	Excellent	1.0	0
XAN 112	Small black	Good	1.2	0
RAB 311	Small red (M)	Excellent	1.0	6
Purdue 6A	Small red	Excellent	1.0	0
Purdue 22	Small red	Excellent	1.2	0
G02333	Small red	Excellent	1.2	0
Carolina	Small red	Good	1.2	0
F0060	Small red	Excellent	1.2	6
APN 83	Small red	Excellent	1.0	0
APN 117	Small red	Excellent	1.0	0
DOR 164	Medium red (M)	Excellent	1.2	0
Linden	Large red (A)	Excellent	1.2	0
Higuerillo	Small cream mottled (M)	Fair	1.0	0
Olathe	Medium cream mottled (M)	Good	1.2	0
A 330	Medium cream mottled	Good	1.4	0
Garrapato	Medium cream mottled	Fair	1.2	0
Pinto UI 114	Medium cream mottled	Good	1.0	0
NY79-3939-1	Medium cream mottled	Excellent	1.0	0
2-4-1	Medium white (M)	Excellent	1.2	0
Sutter Pink	Medium pink (M)	Fair	1.2	6
Enxofre	Medium dark yellow (M)	Good	1.0	0
Amarelo Braga	Medium dark yellow	Excellent	1.2	0
UPR 9173-52	Medium grey (A)	Good	1.0	0
A 70	Medium striped (M)	Good	1.0	0
DICTA 09	Small red (M)	Fair	4.4	100
Purdue 5A	Small red	Good	3.8	100
F0057	Medium red (M)	Excellent	4.0	100
KID 3	Medium red	Excellent	3.8	100
Provider	Medium red	Good	4.6	100
UPR 8953-16	Small red mottled (M)	Good	4.4	100
RH7-23	Small cream (M)	Fair	4.0	100
Line 226	Medium white (M)	Fair	4.0	100
INIAP 403	Medium cream (M)	Fair	4.4	100
Calima	Medium red mottled (A)	Fair	4.0	100
G12470	Medium red mottled	Fair	3.8	98
Linea 24	Medium red mottled	Excellent	4.0	98
Pompadour N	Large red mottled (A)	Good	4.6	98
DOR 510	Large red mottled	Fair	4.0	97
87 VA-1153	Large striped (A)	Fair	3.8	97
F0044	Small red	Fair	4.0	97
87 VA-1145	Medium red mottled	Excellent	3.8	96
PVA 1272	Medium red mottled	Fair	3.6	96
Carmine	Large red (A)	Fair	4.0	96
CAN 87	Medium light yellow (A)	Fair	4.0	96
PR-JB-655-F-H	Large red mottled	Fair	4.0	96
LAS 54	Large red mottled	Fair	4.0	93

Table 2. (Continued.)

Accession	Market class[a]	Nodulation[b]	Visual score	KIM5s nodule occupancy (%)[c]
DOR 511	Large pink (A)	Good	4.0	92
CAL 27	Large red mottled	Fair	3.6	91
DOR 513	Small red	Fair	4.0	90
87 VA-1147	Medium red mottled	Fair	4.0	90

[a] M = Middle American origin; A = Andean origin.
[b] Excellent = >80 pink nodules; Good = 41–80 pink nodules; Fair = 21–40 pink nodules (CIAT, 1987).
[c] Values represent the mean occupancy of a total of 50 nodules taken from 10 plants.

Table 3. Relationship between chlorosis rating and nodule occupancy among *Phaseolus vulgaris* L. accessions

Chlorosis rating	Nodule occupancy	Number of accessions
1	0–25%	24
	26–50%	0
	51–75%	0
	76–100%	0
2	0–25%	0
	26–50%	0
	51–75%	0
	76–100%	0
3	0–25%	0
	26–50%	0
	51–75%	0
	76–100%	0
4	0–25%	9
	26–50%	5
	51–75%	10
	76–100%	31
5	0–25%	0
	26–50%	0
	51–75%	0
	76–100%	3

occupancy 0–30%) and were from the Honduran collection. These lines had yellow leaves, scoring 4 or 5 in the foliar assay with KM6001, but were preferentially nodulated by the indigenous strains. These accessions showed N-deficiency symptoms in spite of being well-nodulated by indigenous rhizobia.

Twenty-two of the 24 accessions with very low preferential nodulation by KIM5s, determined by foliar assessment and nodule occupancy, belong to market classes from Middle American origin; the other two (Linden and UPR 9173-52) are from Andean origin (Table 2). From the 26 accessions with very high preferential nodulation by KIM5s, 11 belong to Middle American market classes and 15 are from Andean classes.

Discussion

In this paper we describe the initial step in studying the genetic basis for *Rhizobium* strain preference in leguminous plants. We developed a rapid assay to identify germplasm that is preferentially nodulated by an inoculum strain, KIM5s, or by indigenous soil strains of *R. etli* in competition with KIM5s. The assay is based on inoculation with a Fix^- mutant of KIM5s. In general, when the strain was dominant in the nodules of a plant, the leaves of the plant turned yellow due to N-deficiency. If the Fix^+ indigenous strain dominated in the nodules, then the leaves turned green. We found a significant relationship between leaf color and nodule occupancy by the Fix^- strain in this assay. This assay has powerful applications for the study of nodulation competitiveness because it initially requires a simple visual screen rather than the laborious identification of each nodule occupant. However, results from such a screen should be corroborated with nodule occupancy studies on a subset of the treatments, as we report here (Table 2). The work reported here focused on occupancy of nodules at the crown, and future work will determine whether this reflects occupancy of all of the nodules of the plant (McDermott and Graham, 1989; Vikman and Vessey, 1993).

Using the visual assay followed by selective nodule occupancy studies, we identified common bean accessions that are preferentially nodulated by KIM5s and accessions that are preferentially nodulated by the indigenous strains. Both nodulation preferences appear to be distributed across all market classes of common beans. However, the preference for the indigenous strains appears to be more common among

Middle American accessions (Table 2). A similar tendency was found by Kipe-Nolt et al. (1992) when Middle American accessions were inoculated with a *R. etli* strain isolated from the same geographic region.

In common bean production, the indigenous strains that are poor in nitrogen fixation often exclude high nitrogen fixing strains from the nodules, thereby preventing maximum benefit from being obtained from nitrogen fixation. The genetic variation for preferential nodulation suggests that this trait can be manipulated for agronomic gain. Cultivars that preferentially nodulate with KIM5s can be bred through recurrent selection schemes to enhance nitrogen fixation in the field. This approach to the competitiveness problem will be more widely applicable than previous work that led to the identification of a soybean gene responsible for exclusion of the major *Bradyrhizobium* serogroup in the upper Midwest of the U.S. if preferential nodulation with KIM5s is effective in the presence of diverse indigenous strains. This remains to be tested.

The work reported here lays the groundwork for fundamental studies of the genetic contribution of the plant to the nodulation competitiveness phenomenon. Most previous work on the plant genes involved in nodulation specificity has focused on qualitative traits (Cregan et al., 1989; Dowling et al., 1987, 1989). Since the genotypes described here are all nodulated by both KIM5s and the indigenous strains in the absence of the competitor, the genes identified in these common bean lines should provide insight into the quantitative phenomenon of strain preference. This work will contribute to our understanding of the *Rhizobium*-legume symbiosis and plant-microbe interactions in general.

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