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Enhancing crop legume N_2 fixation through selection and breeding

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Table of contents

Abstract
Introduction
Operational framework for selection and breeding of legumes for enhanced N_2 fixation
Strategies for selection and breeding of legumes for enhanced N_2 fixation
Host × strain specificity
Measuring N_2 fixation
  N yield
  N difference
  ^{15}N methods
  Xylem N solutes
  Acetylene reduction assay (ARA)
Assessing heritability and repeatability of N_2 fixation
Research to select and breed for enhanced N_2 fixation in legumes
  Common bean
    Soybean
    Studies to define genetic variation in N_2 fixation
    Improvement / breeding programs
  Soybean
    Studies to define genetic variation in N_2 fixation
    Improvement / breeding programs
    Controlled nodulation — selection and breeding
Conclusions
References

Abstract

Legume N_2 fixation is variable, but nonetheless is a valuable process in world agriculture. There is great potential to increase the contribution by the crop legumes to the world's supply of soil N. This will be achieved by (i) increasing the area of legumes sown by farmers; (ii) improved management of the crops in order that the major determinants of productivity, e.g. land area, water availability, are converted to harvested product with maximum efficiency; and (iii) genetic modification of the commonly-grown species to ensure high dependence of the legume crop on N_2 fixation at all levels of productivity. Currently-used methods for measuring N_2 fixation and for assessing heritability and repeatability of N_2 fixation in breeding and selection programs are reviewed. Results from research programs to define genetic variation in N_2 fixation and to enhance N_2 fixation through selection and breeding are presented with particular emphasis on common bean (*Phaseolus vulgaris*) and soybean (*Glycine max*).

Introduction

The demand for nitrogen (N) in world agriculture is increasing at a rate approximately equivalent to the rate of increase in world population, i.e. about 2% p.a. (FAO, 1992a). However, the amount actually available and consumed does not increase at this rate because of the continuing rundown of the N-supplying capacity of agricultural soils, the lack of purchasing power of impoverished communities for production commodities such as nitrogenous fertilizers and the knowledge gaps between researchers and farmers in N management of crops and soils for maximum production efficiency.

In 1992, 905 mill. ha were used globally to produce 2,228 mill. t grains and oilseeds (Table 1). We calculate that about 80 mill. t N was required (consumed) to achieve this level of production. The sources of supply were fertilizer (53 mill. t reduced through losses to 40 mill t. [48% of total consumed]), mineral N from soil sources (31 mill. t or 37%) and plant N fixed via biological nitrogen fixation (BNF) by the pulse and oilseed crop legumes (12 mill. t, or 15%). Of the 31 mill. t N supplied to grain and oilseed crops from the soil, a substantial proportion would be recycled legume residue N. Thus, legumes play a key role in the maintenance of world food and fibre production. The value of N consumed annually for global grain and oilseed production is about US$33 billion (costing N at US$400 t^{-1}). Of this amount, N_2 fixation by the crop legumes supplies US$5 billion worth of N. A 15% improvement in N_2 fixation equates to almost US$1 billion additional N.

The options for improving N_2 fixation are two-fold: management of the legume to maximize growth and minimize stresses (Peoples et al., 1994), and breeding legumes with enhanced capacity for N_2 fixation. The potential for the latter was recognized at least two decades ago (Lie and Mulder, 1971; Phillips et al., 1971). However, progress has been slow. Mytton (1983) noted that little attention had been given to an operational breeding approach to improve N_2 fixation and that the basic genetic information necessary for understanding the expression of desired characters was largely absent (see also Graham and Temple, 1984). To some extent, those observations remain relevant today.

Our foci in this review are the practical 'operational' aspects of selecting elite N_2 fixing genotypes and incorporating genes for enhanced N_2 fixation into other (agronomically desirable) backgrounds through breeding. We pay particular attention to just two species — common bean (*Phaseolus vulgaris*) and soybean (*Glycine max*). They have been subjected to disproportionate research in the area of N_2 fixation improvement, probably for different reasons. In the case of the common bean, the economic importance of the crop as a pulse legume, particularly in Latin America, together with low N_2 fixation activity prompted interest and subsequent research. Soybean, on the other hand, is the most widely-grown legume and enhancement of N_2 fixation of this crop would have very large economic benefits.

Operational framework for selection and breeding of legumes for enhanced N_2 fixation

Bliss (1993) suggested that selection and breeding for enhanced N_2 fixation should be done under conditions of low soil N to allow expression of N_2 fixation potential and discrimination between high- and low-fixing lines. Then the following would form the basis of an operational framework for the breeding program:

- choice of traits (characters) as selection criteria that can be measured precisely and economically, while

Table 1. Global statistics of area and production of crops in 1992 and estimates of amounts of N consumed and supplied from various sources

Crop	Area[a] (mill. ha)	Production[a] (mill. t)	N (mill. t)			
			Consumed[a]	Supplied		
				Fertilizer[b]	Soil[c]	BNF[d]
Cereals	700	1,950	60	50	22	-
Oilseeds	83	107	4	3	2	-
Soybean	55	114	14	0	6	8
Pulses	67	57	5	0	1	4
Total	905	2,228	83	53	31	12

[a] Amounts N required for each tonne grain produced were as follows: cereal - 30 kg N t^{-1}; oilseed - 40 kg N t^{-1}; soybean - 120 kg N t^{-1}; pulses - 80 kg N t^{-1}.

[b] FAO (1992a, b).

[c] Calculated by difference, assuming 75% fertilizer-N ends up as plant-available soil nitrate-N.

[d] Assume average Pfix for soybean = 60%; average Pfix for pulses = 80%.

allowing discrimination between superior and inferior lines;
- variability in legume germplasm and heritability of differences for either Pfix (proportion of plant N derived from N_2 fixation) or other N_2 fixation traits;
- identification of genetically diverse parents, incorporating both agronomic and N_2 fixation traits;
- choice of selection units (i.e. individual plants or families) that facilitate precise quantification of traits of interest and allow production of progeny from selected plants;
- use of a breeding procedure (e.g. mass selection, family selection) that provides maximum genetic gain for N_2 fixation and recombination with other (agronomic) traits.

Strategies for selection and breeding of legumes for enhanced N_2 fixation

Nutman (1984) concluded that high N_2-fixing lines of the temperate forage species, red clover (*Trifolium pratense*), were superior because of an enlarged N_2 fixing system, rather than because of increased efficiency of N_2 fixation. Superior plants had the following characteristics: earlier nodulation, more nodules and larger nodules. Significantly, there were no differences between the high N_2-fixing lines and control lines in indices of nodule efficiency, i.e. plant dry matter, plant N and ARA (acetylene reduction assay) per unit nodule mass. Increased nodulation may be the key to enhanced N_2 fixation of a number of the crop legumes, although the efficiency of nodule function, rather than nodulation per se, may be critical for certain species, e.g. common bean, and particular situations, e.g. soybean in the mid-west of the US. Thus, the general strategies for increasing legume N_2 fixation are aimed at:

- maximizing legume yield within the constraints imposed by agronomic and environmental considerations. This approach has particular application to low-yielding species such as common bean, lentil (*Lens culinaris*), mung bean (*Vigna radiata*) and chickpea (*Cicer arietinum*). It has less relevance for the larger, vigorously-growing species like fababean (*Vicia faba*), pea (*Pisum sativum*) and soybean (Attewell and Bliss, 1985; Herridge and Bergersen, 1988; Jensen, 1986). Increasing legume yield can be achieved by plant breeders in traditional breeding programs. Biological yield largely determines N_2 fixation, particularly in low N soils (Duc et al., 1988; Hardarson et al., 1984; Kumar Rao and Dart, 1987). Breeders, however, normally select for grain yield, rather than for biological yield. Grain yield, the product of biological yield and harvest index, is to a degree dependent upon biological yield. Therefore, breeders who operate in low N soils and select for high grain yield will also select for high N_2 fixation.
- active selection and breeding for symbiotic characteristics in legumes. Examples of these are nitrate tolerance, i.e. the ability of the plant to nodulate and fix N_2 in the presence of soil nitrate, and general nodulation capacity. Natural variation for nitrate

tolerance and nodulation capacity exists and has also been created using plant mutagenesis (Betts and Herridge, 1987; Carroll et al., 1985a, b; Herridge and Betts, 1985, 1988; Jacobsen and Feenstra, 1984; Park and Buttery, 1988). It may be impossible, and even undesirable, to produce a legume that is solely dependent upon N_2 for growth and cannot use nitrate; there is scope, however, to improve the levels of tolerance for the majority of the commonly-grown crop legumes.

- optimizing the numbers and effectiveness of rhizobia in the rooting zone, through strain selection and inoculation techniques, and through plant breeding for promiscuous or selective nodulation (Cregan and Keyser, 1986; Devine, 1984; Kueneman et al., 1984). Continued improvements in the effectiveness of strains of rhizobia used as legume inoculants and in the process of inoculation should also be sought (Brockwell and Bottomley, 1994). There appears to be scope to use strains of rhizobia for specific environmental niches, e.g. acid tolerant strains for acidic soils (Howieson and Ewing, 1986).

Host × strain specificity

Accounting for host × strain specificity when selecting and breeding for enhanced N_2 fixation presents practical problems (Mytton, 1984). There are essentially two approaches. The first approach is to consider host × strain specificity important and to identify highly effective combinations of host cultivar and rhizobial strain (Alwi et al., 1989; Mytton, 1975, 1983; Mytton et al., 1977; Nambiar et al., 1984). An extension of this approach can be found in the program at USDA Beltsville which aims to develop cultivars of soybean that bypass the native soil rhizobia and only nodulate with highly effective inoculant strains (Cregan and Keyser, 1986).

The second approach is to ignore specificity and to screen plant genotypes in a field soil containing high numbers of effective rhizobia or screen under glasshouse or field conditions using strain(s) known to be highly effective with a wide range of genotypes (Betts and Herridge, 1987; Bliss and Miller, 1988; Herridge and Rose, 1994; Kueneman et al., 1984; Nangju, 1980; Phillips and Teuber, 1985). This approach has merit. In the majority of field situations and with current inoculation technology, it is not possible to control the mix of rhizobial strain(s) that nodulate a legume crop, making it difficult to establish the highly effective, specific host-strain combination. There is also substantial evidence that a superior host genotype, selected on the basis of N_2 fixation with one or more highly effective strains, will express that superiority with other strains (Buttery and Dirks, 1987; Pacovsky et al., 1984; Phillips and Teuber, 1985; Rennie and Kemp, 1983b; Wiersma and Orf, 1992; Wolff et al., 1991).

Therefore, the protocol for a breeding program that does not aim to produce specific host-strain combinations would involve screening plant genotypes against the most effective strain(s) available. The strains would constitute a native soil population or would be used as inoculants. The latter strain(s) should also have proven effectiveness with a wide range of genotypes of the particular legume. This strategy should allow maximum expression of N_2 fixation by the host and does not involve the major problem of competition for nodulation between inoculant strain(s) and native soil rhizobia.

Measuring N_2 fixation

There is no single correct method for measuring N_2 fixation by legumes. None of the current methods, N yield, N difference, ^{15}N, acetylene reduction and xylem solute (ureide), can be relied upon to provide an accurate measure of N_2 fixation for every legume species grown under all possible variations of soil type and environment. Each method has unique advantages and limitations. Indeed, each method has been used at various times for assessing variation in N_2 fixation amongst legume genotypes and for identifying elite lines in breeding programs (Table 2). There is a trend with time, however, for the acetylene reduction assay (ARA), commonly-used in the 1970's, to be replaced by more accurate and reliable methods. Following is a short description of current methods.

N yield

The simplest estimates of N_2 fixation are obtained by measuring the amount of N in the legume biomass and are based on the assumption that the legume derives all of its N from N_2 fixation. In virtually all cases involving field-grown plants, the values obtained will overestimate N_2 fixation because the method ignores the contribution of soil N to plant growth. In soils that are extremely low in plant-available N, valid compar-

Table 2. Summary of methods used for plant culture and assessing symbiotic activity in programs to select and breed for enhanced N_2 fixation

Species	Program	Plant culture	Assessment		Reference
			Nodulation	N_2 fixation [a]	
Alfalfa	Screening /breeding	Glasshouse pots, 0 nitrate	Score	ARA, shoot, root DM	Seetin and Barnes, 1977
White clover	Screening	Controlled environ. pots, 0 nitrate	-	shoot DM	Mytton, 1975
Pea	Screening	Controlled environ. pots, 0 nitrate	Leghaem-oglobin	ARA, shoot DM	Hobbs and Mahon, 1982
Red clover	Breeding	Controlled environ. test tubes, 0 nitrate	Time, number, size, mass	ARA, shoot DM, %N, N	Nutman, 1984 (synthesis of 25-year program)
Alfalfa	Breeding	Glasshouse pots, 0 nitrate	Score, nodule enzymes	ARA, shoot, root DM	Barnes et al., 1984 (synthesis of 9-year program)
Alfalfa	Breeding	Glasshouse pots 0 and 8 m*M* nitrate; field, low nitrate	Number, flavenoids	shoot DM, %N, N, ^{15}N (I.D.)	Phillips and Teuber, 1985 Teuber and Phillips, 1988
Common bean	Selection /breeding	Glasshouse pots, 0 nitrate; field low/mod nitrate	Mass, number, carbon	ARA, shoot DM, N	Graham and Temple, 1984 (synthesis of 10-year program)
Common bean	Breeding	Field, low nitrate	Mass, number	ARA, shoot DM, N, grain yield, ^{15} N (I.D.)	McFerson et al., 1982 Attewell and Bliss, 1985
	Breeding	Field, low nitrate	Mass, number	shoot DM, N, grain yield, ^{15}N (I.D)	Bliss, 1993 (synthesis of 15-year program)
Common bean	Selection	Controlled environ. pots, 0 nitrate; field, low/mod nitrate ± fert. N	-	^{15}N (I.D.), ^{15}N (nat. abund.) shoot DM, N	Rennie and Kemp, 1983a, b
Soybean	Screening /breeding	Glasshouse pots, 0 and 5 m*M* nitrate; field, + fert. N	Mass, number, enzymes	ARA, ureides - stem, leaves, xylem. shoot DM, N	Wu and Harper, 1990, 1991
Soybean	Screening	Field, mod. soil nitrate	Occupancy grain yield, N N uptake, NHI	shoot DM, N	Leffel et al., 1992
Soybean	Screening	Glasshouse pots, 0 and 2.5 m*M* nitrate field, low and high nitrate soils	Mass, number	ureides - xylem, stem and root. ^{15} (nat. abund.)	Betts and Herridge, 1987. Herridge and Betts, 1988. Herridge et al., 1990.
	Breeding	Field, low and high nitrate soils	-	ureides - xylem F2 - single plant, non-destructive	Herridge and Rose, 1994
Soybean	Breeding	Field, ± fert. N, low/mod. nitrate soil	Mass, number	ureides - xylem ^{15}N (nat. abund.)	Song et al., 1995

[a] ARA - acetylene reduction assay, I.D. - isotope dilution, nat. abund. - natural abundance.

isons of treatment effects may be possible. Although absolute estimates of total N_2 fixed will still be high in these cases, the error may be negligible particularly if total biomass N is large.

N difference

A true measure of N_2 fixation based on legume N yield can only be obtained when the contribution of soil N to total biomass N is determined. This is usually achieved

by growing a non N_2-fixing crop concurrently in the same soil. The difference in total N accumulated by the legume (N_{leg}) and non-fixing control (N_{nonfix}) is regarded as the amount of N_2 fixed. Thus:

$$N_2fixed = N_{leg} - N_{nonfix} \quad (1)$$

The major assumption of the method is that the legume and non-fixing control take up identical amounts of N from the soil. Because of this, the choice of the control is of utmost importance. Ideally, the legume and control should explore the same rooting volume, have the same ability to extract and utilize soil mineral-N, and have similiar patterns of N uptake. The non-fixing control may be a non-legume, an unnodulated legume or a non-nodulating legume, preferably an isoline of the test legume. Unfortunately, there are often substantial differences between N_2-fixing and non-fixing plants in their capacities to use soil N. Even when a supposed ideal non-fixing control is used, e.g. a non-nodulating isoline, erroneous estimates of N_2 fixation may still result from differences in root morphologies (Boddey et al., 1984).

The observation that levels of soil mineral-N were invariably higher following a legume crop than after a non-legume (Doughton and McKenzie, 1984; Evans et al., 1985) led Evans and Taylor (1987) to propose a modification of the N-difference equation to account for differences in the utilization of soil mineral-N by the legume and non-legume (non-fixing control). Additional measurements are made of the amounts of soil mineral-N in the root zones of the two crops at maturity. Thus:

$$N_2fixed = (N_{leg} - N_{nonfix}) + (SoilN_{leg} - SoilN_{nonfix}) \quad (2)$$

With both the N difference and modified N difference methods, greater accuracy will always be achieved when plant-available soil N is low and legume biomass N is high.

^{15}N methods

The stable isotope ^{15}N occurs in atmospheric N_2 at a constant 0.3663 atom% ^{15}N. If ^{15}N enrichment (abundance) in plant-available soil N is different from that in atmospheric N_2, then the proportion of legume N derived from each source can be measured by the isotopic abundances in the legume and in a non-fixing control totally dependent on the same soil N. In many cases, the very small differences in the natural abundance of ^{15}N between plant-available soil N and atmospheric N_2 can be used, provided the samples can be analysed with a very precise mass spectrometer. More usually, the difference between the ^{15}N enrichment of the soil and atmosphere is expanded by incorporating ^{15}N-labelled materials in the soil.

^{15}N enrichment

The ^{15}N enrichment method is generally regarded as the standard method for estimating legume N_2 fixation. Its use has greatly increased over the past decade, a fact that is reflected in the extensive list of recent reviews on the method (e.g. Chalk, 1985; Danso, 1988; Danso et al., 1993; Ledgard and Peoples, 1988; Witty et al., 1988). However, the high cost of instrumentation to measure ^{15}N plus the expense of the ^{15}N-labelled materials are real constraints to even greater use of the method. Its main advantage is that it provides a time-averaged estimate of Pfix, integrated for the period of plant growth to the time of harvest. Thus:

$$Pfix = 1 - \frac{(atom\%^{15}Nexcess_{legume})}{(atom\%^{15}Nexcess_{nonfix})} \quad (3)$$

where, atom% ^{15}N excess = (atom% $^{15}N_{sample}$) — (atom% $^{15}N_{airN2}$), and atom% ^{15}N of air N_2 = 0.3663. The estimate of Pfix is independent of legume yield, although it is necessary to measure dry matter and N yield to estimate the amount of N_2 fixed.

The major assumption of both the ^{15}N enriched and natural ^{15}N abundance methods is that the legume and non-fixing reference plants utilize soil N with the same isotopic composition. With the enriched system, this translates into the legume and non-fixing reference plants utilizing the same relative amounts of N from added ^{15}N and endogenous soil N. This may not always occur and is the major weakness of the method (Witty et al., 1988). Thus, the choice of non-fixing reference plant is of utmost importance. Ledgard et al. (1985) and Witty (1983), amongst others, have shown the effect of the non-fixing reference plant on estimated Pfix. For this effect to be minimized, legume and reference plants should have similar patterns of soil-N use, so that the inevitable shifts in isotopic composition with time and space become inconsequential.

Natural ^{15}N abundance

Almost all transformations in soil result in isotopic fractionation. The net effect is often a small increase

in the ^{15}N abundance of soil N compared with atmospheric N_2 (Shearer and Kohl, 1986). Because the differences are so small, data are commonly expressed as parts per thousand (‰ or $\delta^{15}N$). Thus:

$$\delta^{15}N = 1000\chi \frac{(atom\%^{15}N_{sample}) - (atom\%^{15}N_{standard})}{(atom\%^{15}N_{standard})} \quad (4)$$

where the standard is usually atmospheric N_2 (0.3663 atom%). By definition, the $\delta^{15}N$ of air N_2 is zero. The natural abundance method gives an integrated estimate of Pfix over time and has the advantage of being able to be used in already established experiments, provided non-fixing reference plants are also growing in the experimental plots. Plant N derived from N_2 fixation is calculated thus:

$$Pfix = \frac{(\delta^{15}N_{nonfix}) - (\delta^{15}N_{leg})}{(\delta^{15}N_{nonfix}) - B} \quad (5)$$

The $\delta^{15}N$ value of B is a measure of isotopic fractionation during N_2 fixation and is determined by analysis of the $\delta^{15}N$ of total plant N of the nodulated legume grown in N-free media. Isotopic fractionation during N_2 fixation is minimal but not zero and should be taken into account when calculating Pfix (Peoples et al., 1989a).

Although the principles of the natural abundance method are similar to those of ^{15}N enrichment, the major limitations are quite different. An isotope ratio mass spectrometer capable of measuring accurately differences of 0.1 ‰ (about 0.00004 atom% ^{15}N) is needed. Great care is necessary in sample preparation to avoid isotopic fractionation (see review by Bergersen et al., 1990). As well, contamination by ^{15}N enriched material must be rigorously avoided. The accuracy of the method will depend ultimately on the levels and uniformity of the ^{15}N in the soil. Levels of $\delta^{15}N > 6.0$ are preferable, although values as low as 2 ‰ might still be useful, depending on the level of Pfix (Unkovich et al., 1994). Values below 6.0 are often found in pasture and plantation soils and in natural forest systems (Peoples et al., 1991). Fortunately, for soils that are regularly cultivated, $\delta^{15}N$ values of plant-available N tend to range between 6.0 and 16.0 (Peoples and Herridge, 1990), and can be relatively constant with time and depth (Bergersen et al., 1990). Therefore, the major limitation of ^{15}N enrichment, i.e. choice of appropriate non-fixing reference plant, is less critical.

Xylem N solutes

Collection of xylem sap and analysis of its contents has been used widely for assessing the nutritional status of field-grown plants (e.g. Bollard, 1960). Xylem sap was collected either as sap bleeding spontaneously under pressure from the stump of the intact root following decapitation of the shoot (root-bleeding sap), or under mild vacuum applied to freshly-harvested shoot segments (vacuum-extracted sap). The latter technique (Bennet et al., 1927; Bollard, 1953), facilitated the development of the ureide assay of N_2 fixation from one with restricted application (glasshouse-grown plants, analysis of root-bleeding sap; McClure et al., 1980) to an assay that could be applied to a wide range of species and field environments (Herridge, 1984; Herridge et al., 1987, 1988a, 1990; Herridge and Betts, 1985; Norhayati et al., 1988; Peoples et al., 1989a, b; Rerkasem et al., 1988).

The principal underlying the ureide assay is that the composition of N solutes in xylem sap changes from one dominated by the ureide compounds, allantoin and allantoic acid, in N_2-dependent plants to one dominated by nitrate and amino-N in plants utilizing soil N. In calibration experiments, correlations between the relative abundance of ureide-N (ureide-N as a proportion of total sap-N) in xylem sap and Pfix were extremely strong with regression coefficients of almost unity (e.g. Herridge and Peoples, 1990; Peoples et al., 1989b; Rerkasem et al., 1988). In the case of soybean, the following equations are used (Herridge and Peoples, 1990):

$$Pfix(\%) = 1.56(RU - 7.7)$$
for plants in vegetative and flowering stages (6)

$$Pfix(\%) = 1.56(RU - 15.9)$$
for plants during pod − fill (7)

where the % relative abundance of ureide-N in sap (RU) is calculated as:

$$RU = 400a/(4a + b + c) \quad (8)$$

and a, b and c are, respectively, the molar concentrations of ureides, nitrate and α amino-N (Herridge, 1984).

Table 3. Principal N solutes in xylem sap of N_2-dependent food and oilseed legumes

Amides (asparagine, glutamine)	Ureides (allantoin, allantoic acid)
Chickpea (*Cicer arietinum*)	Soybean (*Glycine max*)
Lentil (*Lens culinaris*)	Pigeon pea (*Cajanus cajan*)
Pea (*Pisum sativum*)	Mung bean (*Vigna radiata*)
Fababean (*Vicia faba*)	Black gram (*Vigna mungo*)
Narrow-leafed lupin (*Lupinus angustifolius*)	Cowpea (*Vigna unguiculata*)
White lupin (*Lupinus albus*)	Common bean (*Phaseolus vulgaris*)
Groundnut (*Arachis hypogaea*)	Winged bean (*Psophocarpus tetragonolobus*)

Not all legumes export fixed N_2 as ureides (Table 3). With the cool season food legumes, around 80% of fixed N_2 is exported from the nodules as the amides, asparagine and glutamine, the remainder as amino acids (Herridge et al., 1988b). It would be extremely useful though to extend the principal of the ureide assay of N_2 fixation activity to the amide exporters. Unfortunately, with these species, none of the readily-measured N solutes appears to be specifically associated with N_2 fixation. There may be scope to measure shifts in asparagine: glutamine ratios, or the relative proportions of nitrate in xylem sap. Calibration experiments involving chickpea, fababean, lentil and pea have been reported (Peoples et al., 1987), but as yet, validation of the relationships under field conditions have not been attempted.

Acetylene reduction assay (ARA)

The acetylene reduction assay arose from observations in the 1960's that the N_2-fixing enzyme, nitrogenase, catalyzed the reduction of acetylene (C_2H_2) to ethylene (C_2H_4). Since that time, the ARA has played a major role in N_2 fixation research because of its rapidity, simplicity, high sensitivity and low equipment and resource costs. The standard ARA method involves enclosing detached nodules or nodulated roots in air-tight containers and exposing them to an atmosphere of about 10% C_2H_2. After an incubation period, gas samples are collected and analysed for C_2H_4 using gas chromatography (Turner and Gibson, 1980). The ARA was adapted for field studies of legume N_2 fixation (Hardy et al., 1968) to mainly compare treatment effects on N_2 fixation, rather than to estimate the amount of N_2 fixed for a particular time interval.

Eventually the ARA method lost favour because of a number of major problems. These included: the difficulty in recovering nodules; the need to interpolate between single, short-term measurements to obtain time-integrated measurements; the need to determine correct conversion ratios between C_2H_4 produced and N_2 fixed, which can vary according to environmental, diurnal and plant effects acting independently on N_2 and C_2H_2 assimilation and reduction; non-linearity in the rate of C_2H_2 reduction over the period of the assay; effects of nodule removal and decapitation of plants and difficulties in sampling, particularly in hard-setting soils. The sheer magnitude of the problems and the resultant errors in estimating C_2H_2 reduction (N_2 fixation) activity suggest that even simple comparisons of material in a breeding program may be invalid. Readers are referred to Turner and Gibson (1980), Witty and Minchin (1988), Vessey (1994) and Minchin et al. (1994) for expanded discussion on the use of the ARA.

Assessing heritability and repeatability of N_2 fixation

In a breeding program, the heritability of desired traits or characters must be established. Low heritability of a character can result from error in measurement or from a complex genetic basis. Unfortunately, most of the published studies which quantified the heritability of N_2 fixation used the ARA method for measurement and there are few examples in which more reliable assessments were made. Notwithstanding this lack of information, it appears that the heritability of N_2 fixation can be readily assessed using standard statistical methods. Furthermore, it appears that N_2 fixation is moderately heritable.

Ronis et al. (1985), using ^{15}N, investigated broad-sense heritability of total and percent fixed N in har-

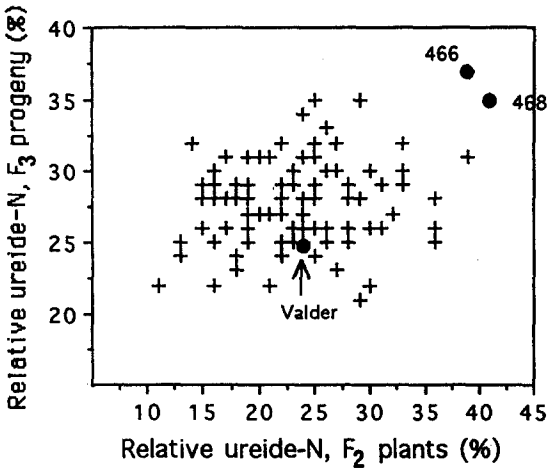

Fig. 1. Relative abundance of ureide-N in xylem sap of 99 individual F_2 plants (+) and their F_3 progeny and low (Valder) and high N_2-fixing (Korean 466 and 468) parents (•) grown in high nitrate soils in successive seasons.

vested seed of three F_2 soybean populations, each of 110 plants. Broad-sense heritability (h^2) was calculated, after utilizing the parental lines to estimate environmental variance, as:

$$h^2 = \frac{\sigma^2_{F2} - 1/2(\sigma^2_{P1} + \sigma^2_{P2})}{\sigma^2_{F2}} \qquad (9)$$

where h^2 = broad-sense heritability,
σ^2_{F2} = variance of the F_2 populations
σ^2_{P1} = variance of the parental (1) population
σ^2_{P2} = variance of the parental (2) population.

Broad-sense heritabilities for fixed N contents of seed ranged from 0.53 to 0.60; estimates for percent fixed N in seed (similar to Pfix) were lower and less consistent, ranging from 0.12 to 0.43. They concluded that improvement of the latter character would be more difficult. However, they didn't concede that the low heritability estimate (0.12) was for progeny of the 'Williams' × 'Calland' cross, both of which had similar, intermediate, N_2 fixation capacity. In other experiments, the parents were more diverse in N_2 fixation, creating greater variation in the progeny. Heritability estimates for those crosses were moderate at 0.37 and 0.43, suggesting that breeding for improved N_2 fixation of soybean using appropriate parents should be possible.

Herridge and Rose (1994) used a similar approach to calculate broad-sense heritabilities of N_2 fixation for 11 F_2 populations of soybean, ranging in size from 14 to 136 plants. Nitrogen fixation was assessed in this study using the xylem ureide technique. Relative ureide-N data for the individual F_2 plants were subjected to analysis of variance to calculate total phenotypic variance (σ^2_P) for the F_2 populations. Single-plant data for the parental and control genotypes was used to estimate environmental variance (σ^2_E). Genetic variance (σ^2_G) was then calculated as ($\sigma^2_P - \sigma^2_E$). Finally, broad-sense heritability (h^2) was calculated as:

$$h^2 = \sigma^2_G/(\sigma^2_G + \sigma^2_E) \qquad (10)$$

Average environmental variance (σ^2_E), calculated from the single-plant values of relative ureide-N for the parent and control genotypes was estimated at 49.4 (Table 4). The resultant estimates of genetic variability ranged from 22.5 to less than zero. Heritability estimates ranged from 0 to 0.31.

Broad-sense heritabilities of single-plant xylem-ureide analysis were assessed also by parent-offspring regression between individual F_2 plants and F_3 progeny means. Heritability of relative ureide-N in xylem saps on a plot basis was calculated from estimations of σ^2_E and σ^2_G from the analysis of variance of the F_3 experiment. The correlation coefficient of the F_2 parent-offspring regression was low ($r = 0.16$) and non-significant ($p>0.05$) (Fig. 1). These results were disappointing but did not necessarily imply that N_2 fixation was not heritable or that N_2 fixation was not quantified accurately. The consistency of assessments of N_2 fixation on a plot basis for parent lines Valder, Korean 466 and Korean 468 over the two years lent support to both the concept of heritability and the ureide method for measurement of N_2 fixation (Fig. 1).

Nitrate levels are variable in field soils. Nitrogen fixation is particularly prone to variations in soil nitrate (Harper, 1987), a response that is more critical when assessing single plants, i.e. F_2 , than when multi-plant samples are used, i.e. F_3 and parent lines in both years. Thus, Herridge and Rose (1994) concluded that, for uniformity in the assessment of single plants for N_2 fixation, it may be necessary to grow plants in sand or water culture and to supply nitrate in measured, nutrient form, rather than grow plants in the field. They concluded also that the F_2 parent-F_3 offspring correlation of 0.16 indicated that selection of individual F_2 plants for enhanced N_2 fixation was better than random. This was supported by the heritability estimate ($h^2 = 0.36$) obtained from partition of variance in the F_3 experiment where relative ureide-N was estimated on a whole-plot basis. Alternatively, assessments for

Table 4. Relative ureide-N in xylem sap at R3-R5 of individual plants of 11 F_2 populations of soybean and 9 parent and control genotypes, grown in a high-nitrate soil

F_2 population/ genotype	No. plants tested	Mean	Range	Phenotypic variance	Broad-sense heritability
		%			
F_2 population					
A	136	27	10–50	63.0	0.22
B	54	27	13–47	67.2	0.27
C	70	28	14–46	71.9	0.31
D	36	28	11–55	65.6	0.25
E	59	28	13–47	58.4	0.15
F	14	26	2–37	36.0	0
G	34	27	15–39	34.6	0
H	102	30	15–55	59.3	0.17
J	110	24	10–48	48.0	0
K	86	29	12–43	37.6	0
L	33	27	14–41	60.8	0.19
Parent and control genotype					
Bragg	20	17	12–32	28.6	-
Bossier	20	18	13–27	12.5	-
Davis	20	22	15–34	21.3	-
Valder	20	24	12–45	70.0	-
Reynolds	20	28	19–34	26.1	-
Korean 464	20	27	16–49	48.0	-
Korean 466	18	39	31–52	113.6	-
Korean 468	20	41	24–60	74.1	-
Korean 469	20	42	30–54	50.3	-

Table 5. Correlation (r) over sites and seasons for relative ureide-N in xylem sap of soybean, pooled for mid and late maturity group experiments

Seasons - site		1989/90 (F_6)		1990/91 (F_7)
		Breeza 1 (low N)	Breeza 2 (high N)	Breeza 1 (low N)
1989/90	Breeza 2 (high N)	0.47**		
1990/91	Breeza 1 (low N)	0.18	0.07	
	Narrabri (high N)	0.19	0.49**	0.14

** Significant at $p = 0.01$, df = 38.

N_2 fixation could be delayed until replication was possible, i.e. in the F_2-derived F_3 generation, or later (see also Bliss, 1993).

In the same program, Herridge and Rose assessed broad-sense heritability of N_2 fixation (relative ureide-N in xylem sap) for F_3-derived F_6 and F_7 lines. Estimates for the F_3-derived F_6 lines ranged from 0.32 to 0.52 and were similar to the value of 0.36, calculated from the F_2-derived F_3 lines. Selection of field sites for evaluation of N_2 fixation was critical for discriminating the high N_2-fixing 'Korean' type of symbiosis. Superiority of the 'Korean-type' genotypes was expressed more consistently in the high-nitrate soils. When correlations of the ureide data for the F_3-derived F_6 and F_7

lines across sites and years were calculated, the results were variable (Table 5).

The 1989/90 data from the low-nitrate (Breeza 1) site was not correlated with either of the 1990/91 data sets (Breeza 1 and Narrabri). However, the 1989/90 data from the high-nitrate (Breeza 2) site was significantly correlated with data from the high-nitrate site in the following season, but not with data from the low nitrate site. Thus, the F_6-F_7 regression analysis gave a similar estimate of heritability or repeatability as the partitioning of variances, provided the high-nitrate sites were being compared. At this level, Herridge and Rose (1994) concluded that progress to selection should be reasonable provided variation of the environmental factors affecting the symbiosis were minimized.

A number of other studies have focussed on the heritability of nodulation, rather than N_2 fixation (see Sinclair et al., 1991). Greder et al. (1986) evaluated three populations of F_3-derived F_5 and F_6 lines of soybean in the field for ability to nodulate with the native soil rhizobia when the seed was not inoculated, and to nodulate in the same soils with a highly effective inoculant strain, *Bradyrhizobium japonicum* USDA 110. Estimates of broad-sense heritabilities for nodulation (nodule mass) by native soil rhizobia ranged from 0 to 0.66 and were equal to or exceeded 0.55 for each population when averaged over sites. For recovery of USDA 110, i.e. nodulation by the inoculant strain, heritability estimates were smaller, ranging from 0 to 0.57. When averaged over sites, they were less than 0.55 for the three populations. These data, together with correlation analysis of nodulation, agronomic and yield traits, indicated that selection for increased nodule mass was warranted.

Similar conclusions were drawn by Arrendell et al. (1985) in a study of F_2-derived F_5 and F_6 generation progenies from a cross of Virginia and Spanish cultivars of groundnut. Broad-sense heritability estimates for nodule number ranged from 0.25 to 0.57 and averaged 0.47 over the two years and six samplings; estimates for nodule weight ranged from 0.48 to 0.78 and averaged 0.66. Estimates for ARA, shoot weight and fruit weight ranged from 0.53 to 0.85. The moderate to high estimates for these traits indicated that superior nodulating and N_2-fixing genotypes within the populations studied could be readily identified and that selection for enhanced N_2 fixation should result in indirect selection for yield.

Research to select and breed for enhanced N_2 fixation in legumes

Common bean

Of all the commonly-grown agricultural legumes, the common bean is regarded as the most inefficient at fixing N_2. This has been established as a result of numerous studies in the US, Canada and countries of South America in which different genotypes of common bean were compared, or where the comparisons were made between common bean and other grain legumes such as cowpea and soybean.

Studies to define genetic variation in N_2 fixation

Graham and coworkers examined more than 600 cultivars of common bean under short-day subtropical conditions. They reported greatest N_2 fixation in the indeterminate, climbing cultivars (Graham and Rosas, 1977; Graham, 1981; Graham and Temple, 1984).

Piha and Munns (1987a) compared N_2 fixation of 9 genotypes of common bean with soybean and cowpea in the field. They also contrasted growth of the three species without fertilizer N (i.e. dependent on N_2 fixation and soil-derived N) with plants that received 220 kg ha^{-1} fertilizer N. Their results showed that the beans did not accumulate dry matter or N or fix N_2 as well as either cowpea or soybean (Table 6). When the bean genotypes were split into maturity groups, it became apparent that there was a maturity effect on the growth and N_2 fixation traits. The early maturing genotypes had the lowest values for both, the late maturing genotypes the highest. Relative N accumulation, an index of the genotype's capacity to satisfy demand for N through N_2 fixation, indicated that the late maturing genotypes of common bean were similar to cowpea and soybean (values of 0.98, 0.97 and 0.94, respectively) and were slightly better than the mid maturity genotypes (value of 0.91). The early genotypes of common bean had by far the lowest value of 0.73. In other words, N_2 fixation could only meet 73% of N demand. Piha and Munns concluded that N_2 fixation was inadequate for the early maturing common bean but adequate for the later maturing types. However, with all maturity types, the capacity for growth and N_2 fixation did not match up to the capacities of either cowpea or soybean.

In a companion glasshouse study, involving 8 of the 13 genotypes grown in the field, Piha and Munns (1987b) confirmed that N_2 fixation by common bean

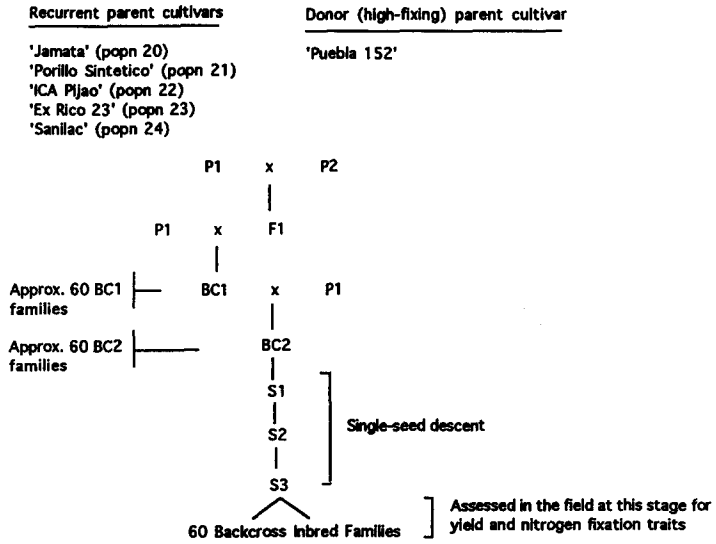

Fig. 2. The backcross inbred method used by Bliss and coworkers to generate populations of common bean with enhanced capacity for N_2 fixation (source: McFerson et al., 1982).

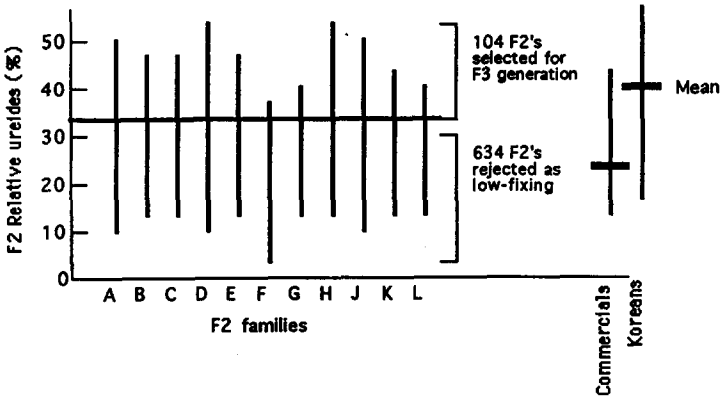

Fig. 3. Ranges of N_2 fixation (relative ureide-N of xylem sap) for the eleven F_2 families and for the commercial and Korean parents. The horizontal line through the families indicates the cut-off point for selection for F_3 generation.

was inadequate but found also that low N_2 fixation activity was not related to low nodulation. They did show, however, that the bean nodules evolved greater amounts of H_2 than those of either soybean or cowpea resulting in reduced efficiency of function (relative efficiency [RE] values of 0.5–0.7 for bean versus 0.95 for soybean and cowpea).

Pacovsky et al. (1984) had previously published RE's of 0.5–0.6 for symbioses of 3 cultivars of common bean and 9 rhizobial strains. They concluded that the copious production of H_2 and the associated energy loss limited the productivity of the bean symbiosis. The RE values indicated that more than 40% of the available electron flow to nitrogenase was used to reduce H^+ to H_2, rather than N_2 to NH_4^+ .

Hungria and Neves (1987), in a factorial study of 5 bean cultivars and 6 strains of rhizobia, endorsed these findings. Their data showed a strong inverse relationship between nodule H_2 production and both nodule specific activity of C_2H_2 reduction, i.e. N_2 fixation, and plant N yield, and confirmed that low N_2 fixation was not a result of low nodulation (Table 7). For example, strain SEMIA 487 produced, on average, 33% more nodules than strain C-05, yet fixed about half the N (shoot N values of 96 versus 183 mg plant^{-1}). Hydrogen evolution and therefore nodule RE were affected

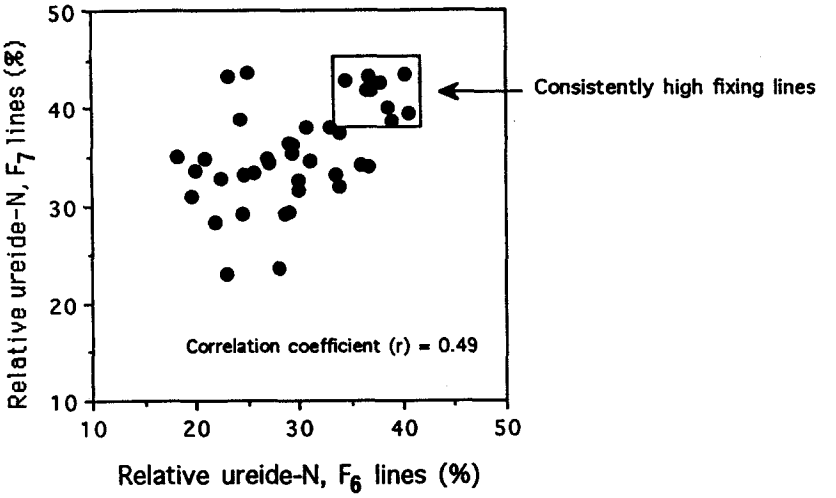

Fig. 4. Relative abundance of ureide-N for F_3-derived F_6 and F_7 lines of soybean, grown in successive years on high nitrate soils at Breeza and Narrabri, Australia.

Table 6. Growth and N_2 fixation of genotypes of cowpea, soybean and early, mid and late maturing common bean in the field in California (data from Piha and Munns, 1987a). Values shown are the means for the following number genotypes: cowpea (2), soybean (2), late bean (3), mid bean (2), early bean (4)

Species	Days to maturity	Total shoot N		Relative N accumulation[a]	N_2 fixed (-N)
		-N	+N		
		(kg ha^{-1})			(kg ha^{-1})
Cowpea	84	254	261	0.97	196
Soybean	100	252	268	0.94	189
Bean - late maturity	96	171	175	0.98	109
mid maturity	90	113	124	0.91	52
early maturity	84	97	132	0.73	35

[a] N in shoots of +N plants/N in shoots of inoculated (-N) plants.

by both plant genotype and rhizobial strain. Cultivar Negro Argel and strain C-05 had RE values approaching the high values reported above for soybean and cowpea.

These were significant findings because a number of programs aimed at improving bean N_2 fixation were based on the premise that nodulation per se was inadequate (e.g. the CIAT program) and that selection for improved nodulation traits would lead to increased levels of N_2 fixation (CIAT, 1987; Kipe-Nolte et al., 1993). The data referred to above suggest, however, that the efficiency with which the nodules function, rather than the gross number or weight of the nodules, is the major problem. Thus, in selection and breeding programs where nodulation is used as the principal criterion for selection, opportunities for worthwhile progress may be limited. A more direct and useful selection trait would be N_2 fixation (or plant N or seed N yield under low N conditions of growth), with number or weight of nodules serving only to confirm that nodulation had occurred.

Hardarson et al. (1993) reported results of a coordinated program, sponsored by the International Atomic Energy Agency (IAEA), to investigate the N_2-fixing potential of common bean and to identify high-fixing lines which could be used as parents in breeding programs. Experiments were conducted in Austria, Brazil, Chile, Colombia, Guatemala, Mexico and Peru during 1985 to 1991.

Table 7. Nodulation (weight, specific activity for C_2H_2 reduction and H_2 evolution, RE) and N yield (N_2 fixation) of bean cultivar and rhizobial strains, glasshouse cultured in Brazil (data from Hungria and Neves, 1987). Values shown are 3 of the 5 bean cultivars, averaged over the 6 strains, and 3 of the 6 rhizobial strains, averaged over the 5 cultivars

Cultivar/strain	Shoot N	Nodulation			
		Weight	C_2H_2 redn	H_2 evolutn	RE[a]
	(mg plant^{-1})	(g plant^{-1})	(specific act. umol g nod^{-1} h^{-1})		
Bean cultivars					
Negro Argel	117	0.54	16.3	2.5	0.85
Venezuela 350	80	0.64	15.3	4.0	0.74
Rio Tibagi	61	0.42	9.8	4.7	0.52
Rhizobial strains					
C-05	183	0.48	25.5	3.1	0.88
SEMIA 487	96	0.64	19.3	4.0	0.79
CIAT 727	22	0.29	9.0	5.1	0.44

[a] RE = 1 - H_2 evolved (air)/C_2H_2 reduced.

Table 8. Summary of N_2 fixation data for common bean, grown in the field in Central and South America and in Austria

Year/location	No.	Range		Commercial cultivar		
	cultivars	Pfix	N_2 fixed	Name	Pfix	N_2 fixed
		(%)	(kg ha^{-1})		(%)	(kg ha^{-1})
1987 - Austria	29	27–67	25–165	-	-	-
1987 - Brazil (i)	17	12–25	4–12	Rio Tibagi	22	8
- Brazil (ii)	7	19–53	11–53			
1987 - Chile	21	38–60	27–62	Tortola	44	50
1988 - Chile	12	27–60	25–115	Tortola	52	90
1985 - Colombia	9	32–47	18–36			
1989 - Guatemala (i)	10	69–73	92–125	ICTA-San Martin	70	105
- Guatemala (ii)	10	22–57	12–50	ICTA-Tamazulapa	31	24
1987 - Mexico (i)	20	5–58	7–108	Flor de Mayo	51	89
1988 - Mexico (ii)	17	0–50	0–70	Flor de Mayo	20	25
1986 - Peru	20	24–56	15–59	Canario Divex	54	42
1988 – Peru	22	3–56	7–51	Canario	47	31

Large effects on Pfix and N_2 fixed were recorded for genotype, location and year of experiment (Table 8). The high values were for adapted cultivars and breeding lines grown under favourable conditions. In four of the experiments, individual genotypes fixed in excess of 100 kg ha^{-1}, through the combined effects of high yield and moderate to high Pfix values (50 to 73%). Conversely, low N_2 fixation, e.g. Brazil (i), tended to

be associated with unfavourable conditions of growth, i.e. high temperatures and dry soil, rather than with inferior genotypes. Average Pfix and N_2 fixed for the commercial cultivars was 43% and 52 kg ha^{-1}, respectively. The participants in the program concluded that the best of the cultivars in each country's trials could be considered for commercial release if all other traits were acceptable. If not, they certainly could be used as high-fixing parents in breeding programs.

Improvement/breeding programs

One program that has made progress in breeding for increased N_2 fixation in common bean is that of Bliss and co-workers at the University of Wisconsin. The genotypes chosen as the recurrent parents were the well adapted, commercially-acceptable cultivars 'Jamapa', 'Porrillo Sintético', 'ICA Pijao', 'Ex Rico 23' and 'Sanilac'. The late maturing, indeterminate, climbing type 'Puebla 152', was used as the high N_2 fixing donor parent (McFerson et al., 1982). Using an inbred backcross method to develop populations of bean which resembled the recurrent parent in desirable agronomic traits but differed in N_2 fixation and yield in low N soils, they produced hybrid lines with enhanced N_2 fixation, acceptable seed yield and other agronomic traits of the recurrent parents (Fig. 2).

The various populations of inbred lines associated with the five recurrent parents (Fig. 2) have been extensively studied during the past decade (see Bliss, 1993). Data for two of the populations are presented in Table 9. Sanilac is a white seeded navy bean, grown extensively in the northern U S until recently. It produced only modest yields and had low N_2 fixation. The inbred lines from the cross of Sanilac and Puebla 152 showed substantial improvements in yield and N_2 fixation, which were combined in some cases with other desirable (agronomic) traits. St Clair et al. (1988) reported lines 24–17, 24–21 and 24–55 fixed 6 to 10 times as much N as Sanilac, showed substantially higher rates of growth and produced up to 40% more grain N. None of the inbred lines could match Puebla 152, the high-fixing donor for plant and seed yield and N_2 fixation. One line, 24–21, also retained the desirable characteristics of short maturity and determinacy. The enhanced yield and N_2 fixation of line 24–17 were linked to the undesirable traits of late maturity and indeterminacy. Line 24–55, although agronomically-acceptable, did not perform as well as 24–21. These results were significant because they indicated that N_2 fixation was heritable and could be combined with other agronomically-desirable traits.

The cross of the black seeded, Porrillo Sintético and Puebla 152, on the other hand, did not produce lines with enhanced yield and N_2 fixation, i.e. superior to Porrillo Sintético (Table 9) (Attewell and Bliss, 1985; St. Clair et al., 1988). Reasons for this could be that the original selection for high N_2 fixation, based on the ARA, was not effective or that the two parents did not differ genetically in N_2 fixation.

Bliss and coworkers used a variety of methods for evaluating and measuring N_2 fixation by the hybrid lines and parents in the breeding program, including plant N yield, seed yield, seed N yield, ^{15}N methods (enriched and depleted formulations), acetylene reduction assay and nodulation indices (Attewell and Bliss, 1985; Pereira et al., 1989; St Clair et al., 1988). They concluded that selection of breeding lines based on plant and/or seed N was most effective and most cost-effective. In their studies, these parameters were always highly correlated with N_2 fixation, determined using ^{15}N (St Clair et al., 1988). The only proviso was that the plants were grown in a low N soil and that the plants were well nodulated by highly effective rhizobia, either introduced by inoculation or already present in the soil.

They did not consider that the xylem ureide method was appropriate for assessing N_2 fixation in the program, mainly because of lack of familiarity with the method rather than for technical reasons (Bliss and Miller, 1988). The xylem ureide method was essentially developed for soybean (Herridge, 1982, 1984; McClure et al., 1980) but was subsequently extended to other crop legumes. In a preliminary experiment, the relative abundance of ureide-N in root-bleeding (xylem) sap of common bean was shown to vary as the plants' proportional dependence on N_2 fixation and mineral N varied (Peoples and Herridge, 1990). These results were confirmed in calibration experiments (Hansen et al., 1993; Peoples and Herridge, unpubl. data). Thus, the ureide method could be used in breeding programs involving the common bean in much the same way as the method was used to assess N_2 fixation by single plants and plots of breeding lines of soybean (Herridge and Rose, 1994).

Bliss and co-workers have now released five high N_2-fixing lines of common bean, designated WBR22–3, WBR22–8, WBR22–34, WBR22–50 and WBR22–55 (Bliss et al., 1989; Bliss, 1993). They are all derived from the cross of ICA Pijao, a black seeded, indeterminate, bush type, bred for sub-tropical conditions,

Table 9. Nitrogen fixation, yield and agronomic traits of recurrent parents Sanilac and Porrillo Sintetico, high-fixing parent Puebla 152, and inbred backcross progeny (mean of 2 experiments) (source: St. Clair et al., 1988)

Parent or line	N yield ($g\ plant^{-1}$)	Pfix[a] (%)	N_2 fixed ($mg\ plant^{-1}$)	Rate of N_2 fixation ($g\ plant^{-1}\ d^{-1}$)	Maturity (days)	Seed N yield ($mg\ plant^{-1}$)
Sanilac	739	7	46	0.6	96	636
24–17	1073	44	475	6.5	107	900
24–21	987	31	306	4.1	93	842
24–55	836	35	292	4.3	100	702
Puebla 152	1222	56	674	6.0	114	1083
Porrillo Sintetico	1197	50	597	8.5	107	991
21–16	1072	49	517	6.2	107	844
21–38	997	49	493	6.9	107	757
21–43	1030	48	491	5.2	107	832

[a] Pfix — proportion of plant N derived from N_2 fixation.

and Puebla 152 (Fig. 2). In field tests in Brazil during 1987, one of the lines (WBR22–34) fixed twice as much N as the standard Brazilian cultivar, Rio Tibagi, and about 20% more than Negro Argel, identified in a previous study as a high fixer (Table 7; Hungria and Neves, 1987). Negro Argel had the highest Pfix value (54%), followed by WBR22–34 (44%) then Rio Tibagi (35%). Line WBR22–3 performed favourably in a field experiment in the same year in Mexico. It had the fourth highest value for Pfix (50%) and the second highest value for N_2 fixed (90 $kg\ ha^{-1}$). Interestingly, the best performing line was 21–58, a line from Porrillo Sintético x Puebla 152 cross. The five released lines outyielded Rio Tibagi consistently in experimental and national yield trials. In 1984–6 trials in Brazil, yields of the lines exceeded those of Rio Tibagi by 10 to 25%.

The five released lines as well as other breeding lines selected for high N_2 fixation and important agronomic traits have undergone further evaluation during the past five years. Bliss cautions that although the lines usually yield well under low N conditions (because of good N_2 fixation), their utility as commercial cultivars will depend just as much on other traits such as disease resistance and seed type (Bliss, 1993). Thus, breeding programs are underway in countries like Brazil to combine traits like disease resistance and high N_2 fixation into adapted breeding lines, using the general protocols outlined over a decade ago (McFerson et al., 1982; Fig. 2).

When considering future directions for breeding of common bean, Bliss (1993) suggested that breeding plants with capacity to nodulate and fix N_2 in the presence of soil nitrate, i.e. nitrate tolerance, should be a priority. There has been very little emphasis on selection and breeding for this character in common bean, even though it has been a major priority for soybean (see below). There is evidence also that variation exists for nitrate tolerance in both natural populations and in mutant lines (Park and Buttery, 1988, 1989; St. Clair et al., 1988) and that common bean is particularly intolerant of the suppressive effects of soil nitrate (George and Singleton, 1992).

Other selection traits that may have merit in a breeding program for common bean are early and late nodulation (Chaverra and Graham, 1992; Kipe-Nolt et al., 1993; Kipe-Nolt and Giller, 1993), although the practicalities of repeated assessments of large numbers of plants for nodulation may prove impossible. The more direct protocol of Bliss (1993) of growing plants in low N soils and measuring dry matter and N yields of plants and grain is far simpler and has the added advantage that the effects of the nodulation traits on accumulation of fixed N by the plant have been effectively integrated by the characters measured.

Table 10. Published estimates of N_2 fixation, crop N and grain yields of soybean

Country	Grain yield (t ha^{-1})	Total crop N (kg ha^{-1})	N_2 fixation		Method of assessment	Reference
			Pfix (%)	N_2 fixed (kg ha^{-1})		
Australia	0.18–3.31	33–302	0–83	0–233	ureide	1
"	2.16–3.96	346–406	34–67	139–204	^{15}N	2
"	n.a.	329	95	312	^{15}N	3
US	1.38–2.76	100–187	13–40	14–75	N diff.	4
"	1.84–2.89	246–273	42–78	116–192	^{15}N	5
"	3.41–4.49	368–387	71–80	263–31 1	Ndiff.	6
Thailand	n.a.	121–643	14–70	17–450	^{15}N	7
"	n.a.	50–188	54–78	27–147	^{15}N	8
"	n.a.	157–251	0–45	0–113	^{15}N	9
Canada	n.a.	150–334	14–62	33–151	^{15}N	10
France	2.10–2.70	147–182	26–38	38–70	^{15}N	11

n.a. — not available; 1. Herridge and Holland, 1992; 2. Bergersen et al., 1985; 3. Chapman and Myers, 1987; 4. Weber, 1966; 5. Vasilas and Ham, 1984; 6. Bezdicek et al., 1978; 7. Rennie et al., 1988: 8. Kucey et al., 1988a; 9. Kucey et al., 1988b; 10. Rennie, 1984; 11. Armager et al., 1979.

Soybean

Soybean is the most widely-grown of the crop legumes with 55 million ha sown in 1992, representing 47% of the total area sown to the oilseed and pulse legumes (Table 1). We calculate soybean to fix 8 million t N annually, worth US$3 billion. A 15% improvement in N_2 fixation equates to increased inputs into world agriculture of 1.2 million t N each year, valued at US$500 million (see also economic analysis of improving soybean and alfalfa N_2 fixation in the US (Tauer, 1989)).

Numerous studies have been reported which show the variation in N_2 fixation of soybean. In those studies summarized in Table 10, total N_2 fixed ranged from 0 to 450 kg ha^{-1}; the range for Pfix was 0 to 95%. Variations in N_2 fixation were partly linked to variations in yield, in turn linked to maturity and other genetic traits and to environmental constraints. Soybean clearly has the capacity to produce large yields (up to 4.55 t ha^{-1} grain and >600 kg ha^{-1} total crop N) and fix large amounts of N in low N soils under optimum environmental conditions. Thus, in contrast to the common bean, improving N_2 fixation in soybean will not result primarily from genetically-based improvements in yield.

Variations in the amounts of N_2 fixed can also be linked to variations in Pfix. When the soils in which the plants are grown have high nitrate fertility, plants use moderate to large amounts of soil N, Pfix is reduced to a low level resulting in low total N_2 fixed. In two of the studies in Table 10, Pfix values of zero were recorded (Herridge and Holland, 1992; Kucey et al., 1988a). There is now substantial evidence that improved N_2 fixation of soybean will result from incorporation of nitrate tolerance into commercial cultivars. Soil nitrate inhibition of nodulation and N_2 fixation can affect other soil and crop factors in a number of ways. During early crop growth, elevated levels of nitrate suppress nodulation and N_2 fixation. With continued presence of nitrate, the crop's reliance upon N_2 fixation can remain low, leading to reduced plant N, seed yield and seed N if the two sources of N (nitrate and N_2 fixation) together can't satisfy the crop's demand (Herridge and Brockwell, 1988; Wiersma and Orf, 1992). Low N_2 fixation can also mean a net loss of N from the soil when the high protein seed is harvested (Herridge and Holland, 1992), and increased need for fertilizer N inputs.

Other approaches for increasing soybean N_2 fixation involve optimizing the numbers and effectiveness of rhizobia in the rooting zone, through strain selection and inoculation techniques, and through plant breeding for promiscuous or selective nodulation (Cregan and Keyser, 1986; Devine, 1984; Greder et al., 1986; Kueneman et al., 1984; Kvien et al., 1981).

Studies to define genetic variation in N_2 fixation

Variation in nitrate tolerance within a large and diverse germplasm collection of soybean (489 genotypes) was reported by Betts and Herridge (1987). In the program, initiated in 1980, plants were assessed for growth, nodulation and N_2 fixation (relative ureide-N in xylem sap and plant parts). The first two cycles of screening involved culturing the plants in sand-filled pots in a glasshouse, supplied with either nitrate-free nutrients or nutrients containing 2.5 m*M* nitrate. A further two cycles were conducted in high-nitrate field soils.

There were large variations in responses to nitrate (Table 11; see also Betts and Herridge, 1987). From the original 489 genotypes, 66 'nitrate-tolerant' lines were identified on the basis of an overall index which combined the three ureide indices and the nodulation value. The second screen was similar to the first in identifying variation and confirmed the consistency of 32 of the original 66 'tolerant' lines. Genotypes of Korean origin displayed higher than average levels of nodulation and N_2 fixation in the presence of nitrate. Of the original 19 Korean lines, 15 (80%) were included in the second screening, and nine (47%) were selected for the third round of (field) screening. Only 5% of the remaining 470 genotypes were selected as high-fixing after the two glasshouse screenings. It became apparent also that substantial differences in tolerance to nitrate occurred within the group of commercial cultivars e.g. Davis and Lee had greater tolerance than Bragg.

In the third year, 40 genotypes were sown into a high nitrate soil in the field (Herridge and Betts, 1988). The genotypes showing the highest levels of nodulation and N_2 fixation under these conditions were all Korean lines (Table 12). They had similiar shoot yields to commercial cultivars, Bragg and Davis, suggesting that increased N_2 fixation reduced their use of soil N. Post-harvest measurements of soil nitrate confirmed this; up to 34 kg ha^{-1} additional N was recovered from the Korean plots immediately after grain harvest compared with the Bragg plots. Seed yield of the Korean lines was, on average, 30% less than that of Bragg, due to a combination of shattering, early maturity and poor agronomic type. Correlation matrices among the indices of nodulation and N_2 fixation and plant growth and grain yield revealed independence between the symbiotic- and yield-related characters. Therefore, the Korean lines appeared to be suitable for use as high-fixing donor parents in a breeding programme with selection for both grain yield and N_2 fixation.

Subsequent comparisons of Korean genotypes, 466 and 468, with commercial cultivars, Bragg and Davis, and mutants of Bragg, nts1007 and nts1116 (Carroll et al., 1985b), at five field sites showed that the Korean genotypes nodulated better than Bragg, Davis and nts1116 and were about equal to nts1007 (Herridge et al., 1990). Values for Pfix, estimated using both xylem ureide and natural ^{15}N abundance methods, were similar for the two Korean genotypes, nts1007 and Davis. Bragg had the lowest values for Pfix, with nts 1116 intermediate between Bragg and the other four. These levels of symbiotic activity of the Korean genotypes were in spite of low plant and seed yields and early maturity.

Results from the four years of screening indicated that differences in nodulation between the Korean genotypes and commercial cultivars were observed only when the symbioses were stressed, i.e. moderate to high nitrate supply in glasshouse sand culture and in the field or low numbers of soybean rhizobia in the field. In the absence of stress, nodulation of the two groups was similar. Thus, enhanced nodulation of the Korean genotypes was not mediated through a loss of the autoregulatory processes that limit nodulation, e.g. nts mutants (Delves et al., 1986, 1987), or through an altered ability to assimilate and metabolize nitrate (Betts and Herridge, 1987), but more likely resulted from more efficient rhizobial infection and/or nodule initiation.

Serraj et al. (1992) also examined a large population of soybean genotypes for tolerance to nitrate. From the initial screening of 158 lines, they selected five 'tolerant' lines and compared them with sensitive cultivar Kingsoy for nodulation, ARA, nodular nitrate reductase activities and ARA response to oxygen. The most tolerant line, Tielingbaime, maintained enhanced nodulation and N_2 fixation (ARA) in the presence of 3 m*M* nitrate, compared with the other 'tolerant' lines and the sensitive Kingsoy (Table 13). The nodulation index was about twice that of Jiling 13 and about 15 times that of Kingsoy. The variation in ARA was similar. Tielingbaime also had the lowest nodule NRA, suggesting that its capacity to maintain N_2 fixation in

Table 11. Mean values for the ureide indices of N_2 fixation and for nodulation of genotypes of soybean screened for nitrate tolerance (2.5 m*M* nitrate-N, supplied with nutrients). A total of 489 genotypes were included in the first screen and 87 in the second. Data shown are for selected groups of high and low-fixing lines (source: Betts and Herridge, 1987)

	Relative ureide-N values (%)			Nodulation index[a]
	Xylem sap	Shoots	Roots	(%)
First glasshouse screen				
High N_2-fixing lines (66)	43	39	35	3.3
Low N_2-fixing lines (9)	10	5	3	1.2
Second glasshouse screen				
High N_2-fixing lines (32)	38	20	29	3.7
Low N_2-fixing lines (2)	15	4	10	1.8
Bragg	16	6	8	1.8

[a] (nodule mass/shoot mass) × 100.

Table 12. Assessments of nodulation, N_2 fixation and yield of selected 'nitrate tolerant' Korean genotypes of soybean and commercial cultivars in a high nitrate soil at Breeza, New South Wales, 1985 (source: Herridge and Betts, 1985, 1988)

Genotype	Nodulation		Pfix	Shoot DM	Grain yield
	Wt (mg plant^{-1})	No plant^{-1}	(%)[a]	(g plant^{-1})	(t ha^{-1})
Nitrate tolerant					
Korean 466	376	34.5	31	45.9	1.6
Korean 468	254	16.8	18	43.3	1.7
Korean 469	176	19.5	22	41.6	1.4
Korean 464	319	16.5	11	48.1	1.5
Commercial					
Bragg	24	2.0	0	39.7	2.2
Davis	40	1.3	0	48.5	2.2

[a] Assessed during mid podfill using the xylem ureide technique (Herridge and Peoples, 1990).

Table 13. Nodulation, N_2 fixation and nodule nitrate reductase activity (NRA) of nitrate tolerant and sensitive lines of soybean, grown in pots supplied with 3 m*M* nitrate (source: Serraj et al., 1992)

Line	Nodulation	ARA		Shoot	NRA	
	index[a]	per plant	sp.act.	dry wt	cytosol	bacteroid
	(%)	(μmol C_2H_2 h^{-1})		(g plant^{-1})	(μmol NO_2 h^{-1} g^{-1} f. wt)	
Tielingbaime	3.0	72.6	53.0	46.2	1.42	0.03
Adepta	2.3	42.0	41.5	43.1	1.40	0.43
Jiling 13	1.6	18.8	29.4	40.8	1.58	1.60
Kingsoy	0.2	4.8	-	32.0	n.m.	n.m.

[a] (nodule mass/shoot mass) × 100; n.m. not measured

the presence of nitrate could be due to both enhanced nodulation and reduced uptake of nitrate.

Plant mutagenesis was first used to generate pea with greatly enhanced nodulation and with a degree of nitrate tolerance (Jacobsen and Feenstra, 1984). Initial studies with soybean at the University of Illinois, USA, focussed on the generation and assessment of induced mutants of Williams soybean for defective nitrate reductase (NR) activity (Nelson et al., 1983; Ryan et al., 1983). Mutants were produced with defective constitutive NR activity but with unaltered N_2 fixation and nitrate tolerance.

Carroll et al. (1985a), working with Bragg soybean, produced 15 ethyl methanesulfonate (EMS)-induced mutants from 2,500 families of M_2 seedlings that formed up to 40 times the number of nodules as the parent and displayed increased acetylene reduction (N_2 fixation) activity in the presence of nitrate. These mutants [termed nts (nitrate-tolerant symbiotic)] were described also as supernodulators because they produced greater numbers of nodules in the absence of nitrate and appeared to be defective in the autoregulatory control of nodulation (Carroll et al., 1985a; Delves et al., 1986, 1987). Two categories of mutants were described, namely intermediate and extreme supernodulators. Genetic analysis indicated that the increased nodulation was controlled by a single Mendelian recessive gene operating, in the case of soybean, through the shoot (Delves et al., 1986; Lee et al., 1991). There was no evidence of host × rhizobial strain specificity affecting expression of the supernodulation trait (Carroll et al., 1985b; Gremaud and Harper, 1989). Similar mutant phenotypes have now been selected from other cultivars of soybean, e.g. Williams (Gremaud and Harper, 1989), Elgin 87 (Buzzell et al., 1990), and Enrei (Akao and Kouchi, 1992).

The initial assessments of nodulation and N_2 fixation of the supernodulating mutants under glasshouse conditions indicated substantial nitrate tolerance compared with the parent cultivars (Table 14). In the presence of either 5 or 5.5 m*M* nitrate, nodulation and ARA were increased in young plants 10 to 20 fold. Yield data, however, indicated that the increased symbiotic activity was associated with a 30–40% reduction in total growth and with restricted root growth (Day et al., 1986; Gremaud and Harper, 1989). Ohyama et al. (1993) subsequently showed that the restricted root growth resulted in a reduced capacity to absorb nitrate.

The apparent contradiction of greatly increased nodulation and N_2 fixation of the mutants coupled with depressed growth suggested inefficiencies of nodule function and raised doubts about the validity of comparing ARA values of mutants and parent cultivars. Indeed Day et al. (1987) reported that the nodules of nts283 had an altered morphology in having less haemoglobin and a smaller infected area and lower specific nitrogenase activity, i.e. on a nodule dry wt basis. They did find, however, that nitrogenase activity per unit bacteroid protein was identical with the parent Bragg. A number of studies indicated that the concentration of N in shoots of the mutants was 30–50% greater than that of the parent, which could partially account for the discrepancy of similar or enhanced AR (nodule) activity and depressed growth (Day et al., 1986; Hansen et al., 1992a; Ohyama et al., 1993).

Subsequent assessments of the soybean mutants over whole growth cycles using more reliable methods for measuring N_2 fixation, e.g. ^{15}N and ureide analysis, indicated partial nitrate tolerance of N_2 fixation and reduced uptake of mineral N (Eskew et al., 1989; Hansen et al., 1989; Wu and Harper, 1991). It is likely that both traits are associated with supernodulation (Hansen et al., 1992b). Hansen et al. (1989) reported a pot study showing that enhanced N_2 fixation of the supernodulating mutants during early vegetative growth, considered to be associated with the characteristically rapid and profuse nodulation, was generally not maintained during reproductive growth. The exception was at the highest level of nitrate (10 m*M*) in which mutants nts 1116 and nts1007 fixed 31% and 250% more N, respectively, than Bragg.

Evaluations of the supernodulators in the field in the US and in Australia have been restricted to just four studies, involving mutants of Bragg and Williams soybean (Herridge et al., 1990; Pracht et al., 1994; Song et al., 1995; Wu and Harper, 1991). Grain yields of the supernodulators in the US studies were reduced by 20 – 33% (Wu and Harper, 1991) and 28 – 41% (Pracht et al., 1994) compared to Williams, the parent cultivar, when averaged over years and treatments. Data on N_2 fixation confirmed results of the glasshouse studies by showing that the enhanced early N_2 fixation of the mutants was not maintained during later reproductive growth. Results indicated also greater tolerance to fertilizer N, but infrequent increases in N_2 fixation on an area basis because the higher or similar Pfix values of the mutants could not compensate fully for whole plant yields that were commonly 20–30% below those of normally-nodulating cultivars. Results of the Herridge et al. (1990) were similar.

Table 14. Nodulation, C_2H_2 (N_2) reduction and growth of supernodulating mutants of Bragg and Williams soybean and their normally-nodulating parents in the presence and absence of nutrient-supplied nitrate

Nitrate/genotype	Nodulation		ARA (umol h^{-1})		Growth	
	No ($plant^{-1}$)	mass (mg $plant^{-1}$)	Plant ($plant^{-1}$)	Sp. Act. (g nod^{-1})	Shoot (g $plant^{-1}$)	Root
Bragg - Australia (source: Carroll et al., 1985a)[a]						
0 nitrate						
Bragg	69	886	1.2	n.d.	21.1	n.d.
nts 382	431	1583	1.0	n.d.	11.8	n.d.
5.5 m*M* nitrate						
Bragg	29	174	0.2	n.d.	40.5	n.d.
nts 382	414	1886	2.1	n.d.	28.5	n.d.
Williams - USA (source: Gremaud and Harper, 1989)[b]						
0 nitrate						
Williams	187	95	5.3	56	0.52	0.32
NOD1-3	473	137	9.8	72	0.32	0.12
5.0 m*M* nitrate						
Williams	7	2	0	0	5.19	1.89
NOD1-3	122	88	2.0	16	3.42	1.13

[a] Harvested 64 days after sowing; growth (whole plant) and nodule mass fresh wt basis; ARA data from second experiment, +N plants suplied with 2.75 m*M* nitrate, harvested at 28 days.
[b] Harvested 21 days after sowing.
n.d not determined.

The experiments reported by Song et al. (1995) are noteworthy (Table 15). They showed higher N_2 fixation activity for the supernodulators, relative to commercial cultivar, Centaur, and either reduced grain yields (original mutants) or equivalent yields (supernodulators derived from crossing the mutants with commercial cultivars). However, cultivar Manark was superior to both supernodulators and other commercial cultivars with a combination of high grain yield and high N_2 fixation activity. The major advantage of the supernodulators was to increase the yield of a following cereal crop, sown immediately after soybean harvests.

Wu and Harper (1990) had previously observed that naturally-occurring soybean lines exhibiting the supernodulation phenotype had not been identified, even though the gene control for the trait was relatively unstable and pressure of moderate to high levels of soil nitrate in plant improvement programs could favour selection of such a phenotype. They concluded that the soybean's N metabolism may not limit production of biomass and grain and therefore nitrate tolerance through supernodulation was of no direct advantage to the plant (i.e. for yield).

Results of Song et al. (1995) indicate that a major advantage of supernodulation may be the rotational or carry-over effect for a subsequent cereal crop. The exact cause of the carry-over effect warrants further study but it is likely associated with higher levels of plant-available N in the soil after grain harvest because of reduced uptake of soil nitrate by the supernodulators together with release through mineralization of greater quantities of N from the legume residues. Assessments of N_2 fixation, using xylem ureide and natural ^{15}N abundance methods indicated some potential for reduced uptake of soil N by the supernodulators, compared with commercial cultivars. Low nitrogen harvest indices (not determined in this study), increased concentrations of N in plant tissues (e.g. Hansen et al., 1992a) and the relatively large nodule weights would contribute to increased N in the residues following growth of the supernodulators. Nodule N alone could amount to 15–20 kg N ha^{-1} which represents a significant proportion of the 20–30 kg N ha^{-1} required for the increased cereal yields. Thus, although the large

Table 15. Nodulation, N_2 fixation (relative ureide-N in xylem sap), grain yield and yields of a cereal crop sown immediately after soybean harvest of normally-nodulating and supernodulating genotypes, relative to commercial cultivar, Centaur (source: Song et al., 1995). Data are summarized from results of 6 years of field experiments in Queensland, Australia

Genotype	Nodulation		Relative	Grain	Yield of subsequent cereal
	No.	Mass	ureide-N	yield	
	(% of centaur)				
Centaur	100	100	100	100	100
Manark	n.d.	n.d.	120	120	102
Bragg	107	113	n.d.	96	91
nts1116	245	236	121	96	131
777–36[a]	n.d.	n.d.	119	102	n.d.
nts1007	338	245	113	84	121
T89238[b]	n.d.	n.d.	115	101	n.d.

n.d. not determined
Absolute values for Centaur were: nodule no. = 28; nodule mass = 380 mg plant^{-1}; relative ureide-N = 43%; grain yld = 2.3 t ha^{-1}; subsequent cereal yld = 3.9 t ha^{-1}.
[a] Line derived from nts1116 × Nessen cross.
[b] Line derived from nts1007 × Bossier cross.

Table 16. Pedigrees of the 11 populations of soybean and numbers of single plants or lines assessed at each generation for either plant and seed traits (F_2-F_7 generations), yield (F_4-F_7 generations) or N_2 fixation (F_2, F_6 and F_7 generations)

Population - pedigree	F_2 single plants 1986/87	F_2-derived in F_3 gen. 1987	F_3-derived in F_4 gen. 1987/88	F_3-derived in F_5 gen. 1988/89	F_3-derived in F_6, F_7 gen. 1989–91
	(No.)				
A. 464 × Valder	161	22	144	36	3
B. Valder × 464	61	6	45	5	0
C. Valder × 466	87	16	89	26	1
D. Valder × 468	49	8	58	14	1
E. Reynolds × 466	72	9	73	25	8
F. Reynolds × 464	14	1	11	1	0
G. 464 × Forrest	38	5	49	10	3
H. Forrest × 469	119	14	112	37	6
J. Bossier × 464	121	6	46	10	0
K. 468 × Bossier	93	12	109	29	10
L. Bossier × 469	34	5	47	7	1
Total	849	104	783	200	33

number of nodules on the roots of the supernodulators are to some degree parasitic on the host (e.g. Hansen et al., 1992c), they may represent a source of N for succeeding crops.

All programs that aim to breed legume cultivars with enhanced N_2 fixation must meet certain criteria. Such criteria for commercial application of the supernodulators include:

- heritability and stability of the nodulation phenotype,
- improved N_2 fixation related to increased nodulation,
- increased N benefits to the soil-plant system,
- grain yields equal to current high-yielding cultivars.

The Song et al. (1995) study indicates that progress has been made towards these goals and the breeding program, conducted by Dr Song at Pacific Seeds in southern Queensland, Australia, is continuing. Similarly, the Williams mutants are currently being backcrossed with high yielding lines at the University of Illinois in an attempt to improve performance.

Improvement/breeding programs

We report details of the breeding program of Herridge and Rose, Australia, in which the four Korean genotypes, 464, 466, 468 and 469 (Table 12) were used as high-fixing parents in crosses with commercial cultivars, Valder (maturity group [MG] IV), Reynolds (MG VI), Forrest (MG VI) and Bossier (MG VIII) (Herridge and Rose, 1994). The breeding protocol differed from that used by Bliss and co-workers with common bean (Fig. 2) in a number of ways: material was screened for the most part in high nitrate, rather than low nitrate soils; the xylem ureide method was used to assess N_2 fixation; initial assessments of N_2 fixation were with individual F_2 plants, although later assessments (F_6 and F_7) involved populations of plants. A summary of activities are presented in Table 16.

Nitrogen fixation was assessed on individual F_2 plants using the xylem ureide method. Sap was extracted from the top half of each plant leaving the lower half to continue growth and to produce seed for harvest (Herridge et al., 1988a; Herridge and Rose, 1994). The relative abundance of ureide-N of the F_2 plants varied between 2 and 55%, indicating segregation for N_2 fixation activity (Fig. 3). There was no evidence of heterosis, in contrast to results reported by Seetin and Barnes (1977) for alfalfa, by Hobbs and Mahon (1982) for pea and by Ronis et al. (1985) for soybean. Average relative ureide-N values for the 11 F_2 populations were surprisingly constant (24 – 29%, equivalent to Pfix values of 13 – 20%) and were between the lower values of the commercial parents (17 – 28%; Pfix values 2 – 19%) and the higher values of 3 of the 4 Korean parents (39 – 42%; Pfix values 36 – 40%). The relative ureide-N value for fourth Korean parent, 464, was 27%, about the same as for Reynolds, the best commercial parent. Although average N_2 fixation activities of the F_2 populations were below those of the best Korean lines, 35 individual F_2 plants displayed equally high levels of N_2 fixation i.e. relative ureide-N > 40%.

The F_2 populations were culled on the basis of N_2 fixation (xylem relative ureide-N value >31%) (Fig. 3), plant type (agronomic rating >2 on a scale of 1 to 6) and seed colour (yellow, green or yellow-green). Evidence of linkages between N_2 fixation and other more easily determined plant characters was also sought. Correlation matrices of these characters showed no such linkages. Problems could have occurred if N_2 fixation was found to be linked to certain traits of the Korean genotypes e.g. black, brown seeds, poor agronomic type. On the other hand, linkage to other more benign traits could have led to simpler procedures for selecting material.

At the commencement of this study, the genetic control of enhanced N_2 fixation in the Korean genotypes was unknown. Major genes had been identified which influence *Bradyrhizobium* compatibility (Caldwell, 1966; Vest, 1970; Vest and Caldwell, 1972) and hypernodulation (Carroll et al., 1985) of soybean. Frequency distributions of relative ureide-N values in each of the 11 F_2 populations were normal, with no evidence of discontinuities in these distributions that would suggest a major gene segregation.

Culling of F_3-derived lines in the F_4 and F_5 generations was based on yield and agronomic traits. It was only in the F_6 and F_7 generations that N_2 fixation was again assessed. A number of the F_3-derived lines were clearly superior and, importantly, stable in N_2 fixation (Fig. 4). In both the F_6 and F_7 generations, these lines had relative ureide-N of around 40%, compared with consistently lower values or more variable values for other lines. A number of the consistently high-fixing lines (A82–3, D22–8, K78–1 and E68–5) were subsequently used as parents in a backcrossing program (I A Rose and D F Herridge, unpubl. data).

The values for yield and N_2 fixation presented in Table 17 summarize the progress made in the first cycle of selection. At the high nitrate sites in the F_6 and F_7 trials, Forrest was obtaining 27 and 33% of its N

Table 17. Days to flowering, seed yield and Pfix values at F_6 and F_7 for lines selected for backcrossing and for high (Korean 468) and low N_2-fixing (Forrest) parents

Line	Original cross	Flowering	Seed yield mean 3 sites		Pfix[a] high nitrate sites	
			F_6	F_7	F_6	F_7
		(days)	(t ha^{-1})		(%)	
Forrest		50	2.67	2.66	27	33
D22–8	Valder × Korean 468	46	2.08	2.29	47	55
A82–3	Korean 464 × Valder	52	2.00	1.70	49	49
K78–1	Korean 468 × Bossier	57	1.91	2.09	46	54
A46–4	Korean 464 × Valder	58	2.24	2.20	51	56
Korean 468		43	0.95	0.58	47	52

[a] Proportion of plant N derived from N_2 fixation, calulated using formula in Herridge and Peoples (1990) from relative ureide-N values of xylem sap, measured during late vegetative growth/flowering.

from N_2 fixation at the time of sampling. By contrast, N_2 fixation by Korean 468 accounted for 47 and 52% of current inputs of N. Lines D22–8, A82–3, A46–4, and E72–3 were outstanding in respect of N_2 fixation, with Pfix values of around 50%. The Pfix value for commercial cultivar, Reynolds, was around 41%, suggesting that this cultivar may already have some of the symbiotic characteristics being sought for commercial cultivars, i.e. tolerance of the suppressive effects of soil nitrate on nodulation and N_2 fixation.

Grain yields of the F_3-derived lines in the F_6 and F_7 generations, although as much as three times larger than yields of Korean 468, could not match those of the highest-yielding commercial cultivar, Forrest. Some lines gave yields comparable to older commercial cultivars such as Bossier. In particular, lines A46–4, K78–1 and D22–8 had average yields across the 6 trials of >2.0 t ha^{-1} (Table 17).

Herridge and Rose concluded that selection of the field site for assessing N_2 fixation was vital for discriminating the high-fixing, nitrate-tolerant lines. The enhanced capacity for N_2 fixation of these lines and of the Korean parents was expressed only when N_2 fixation of the commercial cultivars, e.g. Forrest, was suppressed by the high nitrate soils. Data from the F_6 and F_7 trials support this by showing that correlations across sites and/or seasons were improved when the soils were high in nitrate (see Table 5).

Yield of the high-fixing, nitrate-tolerant material needs to be improved by about 20% before commercial release. A second cycle of selection was commenced in 1991 with crossing of lines D22–8, A82–3, A46–4 and K78–1 with high yielding genotypes. Single seed descent lines were formed as F_4 single plant progeny and more than 1400 lines from six populations were field-tested in F_5 and F_6 trials for phenology, growth habit, lodging, disease resistance, yield, shattering and seed oil and protein. The best lines from those assessments will be evaluated for N_2 fixation in the F_7 generation.

Controlled nodulation — selection and breeding

Legume inoculation with rhizobia and bradyrhizobia is a long-established and successful practice, especially with particular crops in the more technically-advanced countries. Vincent (1965) and others have argued that it is a desirable practice in most agricultural soils throughout the world although Date (1977) cautioned that the need to inoculate was not universal and should be carefully determined for each individual situation. Because of this and because of the exacting technology required for production, distribution and use of inoculants, the practice of inoculation remains the exception rather than the rule (Brockwell and Bottomley, 1994).

In the less developed countries in particular, there would be substantial advantages in growing legumes that were nodulated by highly effective rhizobia already in the soil. In Africa in the 1970's and 1980's, soybean was considered a new crop that required

inoculation for effective nodulation (Nanju, 1980). In response, scientists at the International Institute of Tropical Agriculture (IITA) sought to exploit the fact that small farmers in Nigeria had grown well-nodulated soybean such as local cultivar 'Malayan' for more than 30 years with low inputs and without inoculation (Nangju, 1980). Rapid progress could then be made in establishing soybean as an industry in Africa if 'promiscuous' nodulation could be combined with the yield, quality and disease resistance traits of improved 'American' cultivars.

Nanju conducted field experiments in the late 1970's at two sites in Nigeria in which soybean of Asian and American origin were evaluated for nodulation and yield in the absence of inoculation and when inoculated. The two Asian cultivars, Malayan and Orba, nodulated successfully with the indigenous rhizobia, resulting in effective symbioses (Table 18). They did not respond to inoculation. The US bred cultivars, Bossier and Jupiter, on the other hand, nodulated poorly without inoculation and showed large increases in nodule number and mass and in grain yield with inoculation. The American cultivars had much greater yield potential, particularly when compared with Malayan. The ability of Malayan to nodulate with native rhizobia was confirmed in other experiments in Nigeria and in Tanzania (Nanju 1980).

In other trials conducted by IITA scientists, 400 genotypes were screened for promiscuous nodulation (i.e. effective nodulation without inoculation) on low N soils at five sites in Nigeria. Only 10 of the genotypes were promiscuous nodulators — Malayan, M-351, TGm 120, TGm 119, Obo, Indo 216, Indo 180, Indo 226, Indo 243 and Orba (Pulver et al., 1985). The promiscuous nodulation of a number of these genotypes was confirmed in a pot experiment in which rhizobial isolates from the field sites were used as inoculum. Other promiscuous nodulators were identified in trials in Tanzania, Zambia and the Ivory Coast (Chowdhury, 1977; Kueneman et al., 1984) and, in some cases, had already been used as parents in breeding programs because of their vigour under the local conditions.

The potential of combining promiscuous nodulation and yield traits through breeding was first shown by Chowdhury and Doto (1982). F_3 lines, derived from a Bossier × IH 192 cross segregated for both yield and N_2 fixation (Table 19). Lines 3 and 16 retained the earliness and yield of Bossier together with the promiscuous nodulation and enhanced N_2 fixation of parent IH 192. Lines 1 and 10 were high yielding but segregated for low nodulation and N_2 fixation.

Grafting experiments reported by Pulver et al. (1985) provided further evidence that promiscuous nodulation could be used to genetically enhance N_2 fixation in soybean. The experiments showed that the symbioses between the roots of promiscuous nodulators, Malayan and Orba, and native soil rhizobia could supply the N required for the shoots of high yielding, improved lines, Jupiter and Bossier, to realize full yield potential. Subsequent hybridization of the Asian and US cultivars by IITA scientists successfully combined high yield and desirable agronomic traits with promiscuous nodulation and enhanced N_2 fixation for a large range of progeny (e.g. Table 20).

Methods or assessing nodulation and N_2 fixation of uninoculated breeding material include visual scoring of nodulation, ureide concentrations in leaf tissue and shoot colour and grain and shoot yield (Kueneman et al., 1984).

In other environments where inoculation is an option, populations of infective rhizobia often exist in high numbers in the soil and represent a formidable barrier to the introduction of more effective strains (e.g. Devine, 1984). In the USA, large populations of the soybean bradyrhizobia have developed with cropping so typically less than 10% of nodules on soybean are formed by the inoculant and yield responses to inoculation are rare. In these situations, N_2 fixation and yield may be limited by the low effectiveness of the native soil rhizobia (Greder et al., 1986; Kvien et al., 1981). The more recent study of Vasilas and Fuhrmann (1993) showed that Forrest soybean nodulated with highly-effective strain USDA 122 fixed 29% more N_2 and produced 31% more grain than plants nodulated with the native soil rhizobia.

One approach to this problem was to select genotypes of soybean with affinity for highly effective rhizobial strains. Kvien et al. (1981) screened 1600 genotypes for ability to nodulate with native soil rhizobia in Minnesota, USA, and for ability to preferentially nodulate with a highly effective inoculant strain, USDA 110, in the presence of large numbers of native soil rhizobia. They identified several genotypes which nodulated poorly with the native strains and gave increased recoveries of USDA 110 and increased yields when inoculated with USDA 110. In a subsequent report, however, one of the genotypes which had nodulated most successfully with USDA 110 exhibited nodule occupancy that was no different from the check cultivar (Moawad et al., 1984). Greder et al. (1986) concluded that selection of genotypes with high recoveries of USDA 110 would be difficult and suggested instead

Table 18. Nodulation and yield of Asian and American cultivars, either inoculated or grown without inoculation in the field in Nigeria

Cultivar	Nodulation				Grain yield ($t\ ha^{-1}$)	
	No.		Mass ($mg\ plant^{-1}$)			
	-inoc	+inoc	-inoc	+inoc	-inoc	+inoc
Asian						
Malayan	42	50	175	235	0.59	0.57
Orba	42	43	255	310	1.80	1.88
American						
Bossier	7	47	47	311	1.43	2.33
Jupiter	10	35	86	274	2.12	2.97

Table 19. Yield and symbiotic traits of improved (Bossier) and promiscuously nodulating (IH 192) parent lines and derived lines, grown without inoculation in the field in Tanzania (source: Chowdhury and Doto, 1982)

Line	Nodule mass ($mg\ plant^{-1}$)	ARA ($\mu mol\ h^{-1}$)	Flowering (days)	Seed yield ($g\ plant^{-1}$)
Bossier	2	1.9	40	5.2
Line 3	38	10.3	38	6.8
Line 16	42	10.3	38	11.2
Line 1	14	2.6	38	8.4
Line 10	15	1.2	38	9.0
IH 192	67	6.8	68	4.9

Table 20. Yields ($t\ ha^{-1}$) of soybean lines derived from crosses of improved and promiscuously nodulating genotypes, grown without inoculation in the field in Nigeria (source: Kueneman et al. 1984). Bossier is included as an improved check

Line	- fertilizer N	+ fertilizer N
Promiscuous bred lines		
TG×326–034D	2.55	2.55
TG×330–054D	1.98	2.37
TG×457–060C	2.30	2.47
Improved check		
Bossier	0.90	1.60

that selection of genotypes on the basis of nodule mass may be warranted.

A second approach, initiated during the 1970's and 1980's at the USDA laboratories, Beltsville (USA), was to produce soybean cultivars that were more selective in nodulation. The plant would bypass the resident rhizobia in the soil to be nodulated by selected, highly effective inoculant strains (Cregan and Keyser, 1986; Devine, 1984).

This strategy of exploiting the host's capacity to exclude certain rhizobia from forming nodules had its derivation in the identification of host genes affecting nodulation. Five genes have been identified that restrict nodulation in soybean (Table 21). These host genes control nodulation at the species, serogroup and strain level within the soybean rhizobia (Keyser and Li, 1992). The rj_1 gene, responsible for non-nodulation, has been transferred through conventional breeding to several soybean cultivars for use in inoculation and N_2 fixation research. Dr T E Devine's program at USDA, Beltsville, sought to exploit the rj_1 gene by combining soybean cultivars containing the gene with rhizobia that could overcome its restriction. He had established previously that such rhizobia existed in natural populations in the soil in very low numbers. From examination of 100,000 – 200,000 plants in the field, he isolated around 300 strains of rhizobia and tested them for effectiveness. None of them was particularly effective and the program has been put on hold.

The second program at USDA, Beltsville, involved Drs P Cregan and H Keyser and co-workers. They aimed to improve N_2 fixation of soybean by restricting the plant's capacity to nodulate with the less effective but ubiquitous and highly competitive USDA 123 group of strains (serocluster), thereby leaving the plant free to nodulate with highly effective inoculant or indigenous strains, e.g. USDA 110. Progress achieved has been to:

- identify genotypes (PI 371607 and PI 377578) that restrict USDA 123 under controlled (pot study) conditions and in the field ((Cregan and Keyser, 1986) (Table 22),
- identify genotypes PI 417566 and PI 283326 that, together with PI 377578, restrict nodulation by 20 of 23 strains from serocluster 123,
- identify a specificity between plant genotype and serogroup of the restricted strains,
- determine by classical genetic analysis that the restriction of USDA 123 by PI 377578 and the reduced competitiveness of strain MN1–1c on PI 417566 are dominant traits, similiar to restriction by the Rj2, Rj3 and Rj4 alleles (Cregan et al., 1989a, 1989b).

Later work aimed to determine if the genes responsible for nodulation restriction were independently assorted or closely linked. If the latter were the case, then combining the various restrictions through crossing would prove difficult. There may be other problems which may make the task of breeding a single plant genotype that combines all of the desired levels of restriction (nodulation control) very difficult. For instance: the manufactured genotype may also restrict desirable, effective strains; undesirable, infective strains may build in the soil over time, thereby creating the problem all over again; the expression of the restriction characters influenced by environment (temperature, growth medium), similiar to environmental influences affecting nodulation of pea.

Conclusions

Breeding crop legumes with enhanced capacity for N_2 fixation has been promoted as a worthwhile and economic goal since the 1970's. At a meeting in the US in 1978, Dr Peter Graham presented results of research at CIAT, Colombia, where they had already field tested 600 cultivars of common bean for N_2 fixation and had intensively studied 60 of those. By the early 1980's, a breeding program was in place at CIAT (Graham, 1981). A second program involving common bean was initiated in 1980 by Dr Fred Bliss and collaborators at the University of Wisconsin, USA, using elite material from CIAT. Other programs were also initiated at that time involving a range of species and research organizations, e.g. soybean in Africa and Australia, groundnut in India and the US (see Table 2). From that considerable effort, only a very small number of cultivars have been released with improved capacity for N_2 fixation (Bliss et al., 1989).

There may be two major reasons for this apparent lack of success. Firstly, it is a difficult task to combine a single, desirable trait like N_2 fixation with other agronomic and yield traits. Secondly, techniques for accurate assessment of N_2 fixation by field-grown legumes were not available. In recent years, more use has been made of ^{15}N methods (e.g. St Clair et al., 1988), but this technology has real limitations because of cost and the slow turnaround in sample analysis. In a NSW Agriculture (Australia) program to breed cultivars of soybean with enhanced N_2 fixation, the xylem ureide technique proved invaluable for field assessments.

Table 21. Host genetic control of nodulation in soybean (sources Devine, 1984; Keyser and Li, 1992)

Allele	Phenotype	Reference
rj_1	Non-nodulating with virtually all rhizobial strains	1
Rj_2	Cortical proliferations or ineffective nodules formed by rhizobia in serogroups 6(c1) and 122 with cv. Hardee	2
Rj_3	Small nodule-like structures formed by USDA 33 with cv. Hardee	3
Rj_4	Ineffective cortical proliferations by USDA 61 with cvs Hill and Dunfield	4
Dominant	Rudimentry nodules by USDA 205 with cv. Kent	5

1. Williams and Lynch 1954; 2. Caldwell 1966; 3. Vest 1970; 4. Vest and Caldwell 1972; 5. Devine 1984.

Table 22. Nodule occupancy in two PI genotypes of soybean and Williams, grown in the field (source Keyser and Li, 1992)

Genotype	Percent of nodules occupied by		
	USDA 123	USDA 122, USDA 138	Other
Commercial check			
Williams	76	20	4
Restrictive genotypes			
P1371607	3	89	8
P1377578	5	92	3

The technique was used as a single plant, non-lethal assay at the F_2 stage and for assessing replicated plots in later generations, i.e. in the F_3-derived F_5, F_6 and F_7 generations. For the F_2 individual plants, it was necessary to sample xylem sap from around 800 units (plants) and to complete the chemical analysis of the saps within a month. This was done successfully. This assessment could not have been achieved using any other method. With the application of new molecular techniques and the development of bioindicator systems using reporter genes to provide information on the relative levels of ureides, the ureide assay has the potential to become an even more powerful tool for the selection and breeding of (ureide producing) legumes for enhanced N_2 fixation (Wilson et al., 1994).

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