BIO-SCIENCES AND AGRICULTURE

BREEDING FOOD AND FORAGE LEGUMES FOR ENHANCEMENT OF NITROGEN FIXATION: A REVIEW

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ABSTRACT

Nitrogen fixation in legume - root nodules requires the functioning of genes present in the rhizobia that induce nodule-formation. The plant produces the nodules and the energy required for respiration. Genes in both Rhizobium and the plant are responsible for the efficient use of photosynthates for N_2-fixation and assimilation of nitrogen. Genes from Rhizobium and legume hosts that are involved in the symbiosis are being identified, isolated and cloned, to facilitate the manipualtion of either partner. The amounts of nitrogen fixed by grain-legumes vary appreciably, between and within, species, and are also influenced by environment. With few exceptions, most legumes fix insufficient N_2 to support substantial seed-yields. Deficits between the required N and the combined amounts provided by soil and fertilizer help in estimating the improvements in N_2-fixation which is possible through breeding. Since the symbiosis is a complex process, heritability of traits is weak, and most methods which estimate fixation are destructive; a breeding method that allows selection of replicated families rather than single plants is preferred.

INTRODUCTION

Biological nitrogen (N_2)-fixation is estimated to be 65% of the nitrogen (N) currently utilized in agriculture, and is expected to become increasingly important in future crop-productivity, particularly for sustainable systems (Thomas et al., 1997). It involves the enzymic reduction of N_2 gas to ammonia; this reaction is catalyzed only by certain prokaryotic micro-organisms using the enzyme nitrogenase. Nitrogen-fixing micro-organisms are found in most habitats; the most significant ones, in terms of the amount of N_2 fixed per annum for agricultural purposes, are those found on plant roots. By far the most important bacteria involved in nodule formation are species of *Rhizobium* and

Bradyrhizobium which nodulate different species of leguminous plants. Amounts of N_2 fixed per annum vary with crop, soil- type and husbandry, and are usually in the range of 30-300 kg per ha per annum (Beringer et al., 1988).

The cereal crops always yielded higher when used in rotation with legumes (Doyle et al., 1988; Marcellos, 1984). The wheat crop, when planted after chickpea, gave the yield increase from 0.5 to 2.1 t ha-1 over the crop followed by wheat in Australia (Marcellos et al., 1993). Economic analyses revealed that crop- rotation of over four years, involving chickpea and wheat without any nitrogen fertilizer) gave an increase up to 162% in income, from sequences involving chickpea when compared with continuous wheat (Marcellos et al., 1997). The intercropping of pigeonpea with sorghum increased the yield of sorghum tremendously and the biological nitrogen-fixation enhanced the nitrogen status of the soil, resulting in reducing the use of nitrogenous fertilizer in sorghum crop (Addu-Gyamfi and Ito, 1997).

Symbiosis of legumes and rhizobia results in nodule- formation on host roots. Nodule formation begins with bacteria-plant interaction in the rhizosphere and involves the induction of specific genes in both symbionts (Murphy and Thompson, 1988; Long, 1989). The induction of rhizobial nodulation genes (nod) by host-specific flavonoids, which serve as the signal molecules in specific host legumes, is a very critical stage (Hopper et al., 1995). A number of plant-genes are involved in the process of Rhizobium- associated N_2 fixation. Some of the genes induced during the early stage of infection include Rhizobium-induced peroxidase (rip1) (Cook et al., 1995), and ENOD5 and ENOD12 (Scheres et al., 1990). During nodule-development, there are complex interactions between the host-plant and the rhizobia infecting

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the plant. To understand these interactions, both plant and rhizobial mutants are necessary for studying various stages of nodule formation and function (Miller et al., 1991).

Specific genotype-strain combinations that result in enhanced nodulation, increased N_2 fixation and increased yields have been identified (Caldwell and Vest, 1968; Kvien et al., 1981). The success of these enhanced genotype-strain combinations, in a heterogeneous soil environment, will depend on the survival and competitive ability of the inoculant *Bradyrhizobium japonicum* strain and on the ability of host-plant to preferentially nodulate with the inoculant strain (Greder et al., 1986). Selection of lines having decreased ability of nodulation with native strains, but possessing the ability to recover introduced inoculum-strains at high levels, may be a means by which superior genotype-strain combinations can be successfully utilized in the field (Devine and Weber, 1977). Improved *rhizobia* have been produced by genetic manipulation, but field-performance of improved strains has been disappointing and attributed to lack of competitive ability against native populations (Noel and Brill, 1980). Despite the ability of legumes to fix atmospheric nitrogen, the N_2 derived from fixation alone is seldom sufficient to produce large seed-yields. Plants must rely on additional sources to meet their N-requirements (Harper, 1974; LaRue and Patterson, 1981). The essential role of the host-plant in maximizing symbiotic performance is often overlooked and that role is also poorly understood (Bliss and Miller, 1988).

In cereal (non-legume) crops, bacterial genera *Azospirillum, Azotobacter, Acetobacter, Alicaligens, Bacillus, Enterobacter, Herbaspirillum, Klebsiella and Pseudomonas* have been found as their colonizers (Baldani et al., 1986; Roger and Watanabe, 1986; Cavalcante and Dobereiner, 1988; Berge et al., 1991; You et al. 1991 Malik et al., 1994; Mehnaz et al., 1998). Their beneficial effects have been related to biological nitrogen-fixation and production of pyhtohormones that promote root-development and proliferation for efficient uptake of water and nutrients (Tien et al., 1979; Hartmann et. al., 1983. Haahtela et al., 1990). The use and application of bacterial inoculants as biofertilizers has resulted in improved growth and increased yield of cereal crops (Kapulnik et al., 1981; Boddey et al., 1986; Pereira et al., 1988, Kennedy and Tchan, 1992). The DNA-based and immunological studies are underway on the root-colonization and survival of bacteria in the rhizosphere (Vermeiren et. al., 1996; Mehnaz et al., 1998). The effect of *Azospirillum* inoculation, under different levels of farmyard manure, on N_2 fixation, growth and N-yield of two maize cultivars was studied by Elkomy et al. (1998). They found that *Azospirillum spp.* showed a higher degree of host specificity. Like *Rhizobium* strain selection in legumes, they suggested that numerous strains must be isolated from different origins, characterized, and tested under various environmental conditions of each plant cultivar. Furthermore, (Pishchik et al. (1988) found a significant positive effect on yield of potato cultivars, afer inoculating with *Klebsiella mobilis* strains, increasing the yield 1.2-1.4 times as compared to non-inoculated plants.

Improved capacity for fixing N_2 is an environmentally and socially important development, with potential for reducing N-fertilizer requiremnts for crop-production (Hardy, 1985).

The objectives should be to increase the total amount of plant N_2-fixation, the proportion (%) of N derived from fixation, and the yield resulting from fixed N_2.

STRATEGIES FOR PLANT IMPROVEMENT

Effective improvements through breeding for increased N_2-fixation depend on the amount of genetic variability in the gene pool, relations among plant-traits that either enhance or limit fixation, use of an appropriate method for estimating N_2 fixation, the effectiveness of the breeding method used, and favourable host-plant interactions with strains of *Rhizobium*. The number of bacteria and their nitrogen-fixing efficiency were estimated, using improved and local cultivars in the highlands of Turkey. Native rhizobia, specific to the local landrace, were more abundant than those specific to the improved cultivar, but nitrogen-efficiencies of all isolates were consistently poor. The identification of symbiotically efficient and econologically persistent strains of *rhizobia* were

suggested in order to improve nitrogen-efficienices (Keatinge et al., 1995). Maximum selection-response for increased fixation is not likely to be realized in situations where external N is in abundant supply. However, the breeding objectives may include developing plans capable of fixing N_2, in the presence of abundant N. It may also be desirable to select plant-genotypes that will, in addition to fixing N_2 effectively on poor soils, respond to added fertilizer N. Field plot designs and management and breeding methods used can be modified to accommodate these objectives (Bliss and Miller, 1988).

As symbiotic plant-response is dependent on both the host-plant genotype and the microsymbiont, plant-improvement strategies should therefore consider the rhizobial population to be encountered during commercial production. Devine and Weber (1977) suggested selection of soybean genotypes resistant to indigenous rhizobia, and susceptible only to a specific strain, to be added as inoculant. A contrasting strategy is to select a host genotype, such as many Asian and African soybean cultivars that are modulated excessively by native rhizobia (Nangju, 1980).

(i) Genetic Variability in Host Plant

Indirect estimation methods have been used to identify cultivar variability for N_2-fixation potential in various leguminous crop species. It varies from species to species and vice versa.

Differences in acetylene-reduction activity (ARA) values among breeding-lines were shown to be heritable in common bean (McFerson, 1983), cowpea (Miller et al., 1986), groundnut (Arrendell et al., 1985) and mungbean (Fernandez and Miller, 1985a). Similarly, nodule-mass values and number of nodules per plant were heritable and positively correlated with ARA. It suggests that these traits may be used as criteria for selection in the improvement-programmes.

Ronis et al. (1985) reported that, in two soybean populations, broad-sense heritability estimates ranged from 0.59 to 0.6 0 for fixed N_2 content of the seed, and from 0.12 to 0.43 for percentage total seed N from fixation. In common bean, although

heritability of fixed N_2 was not determined, Attewell and Bliss (1985) found that inbred backcross lines of black bean and white navy bean, selected for increased N_2-fixation by indirect methods, fixed more total N_2 and had derived a larger percentage of total N from fixation. Seed-yields were equal to, or greater than, the standard cultivars.

Some studies of host-plant traits that influence fixation have been made. Path-coefficient analyses of variables associated with fixation in cowpea have indicated that nodule-weight per plant was the main parameter contributking to N_2-fixation activity, while nodule number per plant was important, mainly through correlation with nodule weight (Miller et al., 1982). Yield-component analysis in cowpea (Fernandez and Miller, 1985b) has indicated that nitrogen nutirtion, whether from fertilizer or N_2 fixation, strongly influenced pod-number per plant, and that this component was a reliable indicator of N_2-fixation ability. However, other components (e.g. seed number per pod and mean seed weight) were fairly stable and cultivar-specific and, so, were unaffected by inoculation or N fertilization. They suggested that breeding-efforts to enhance N_2 fixation potential should focus on increasing the pod-production and retention.

When environmental stresses are experienced by legumes, additional plant characteristics may become important. In cowpea, the effect of water-stress on N_2-fixation variables was far greater than the influence of genotype, when those genotypes were selected for relative drought-resistance (Walker and Miller, 1986). Path-coefficient analysis revealed that leaf-water potential was correlated positively with N_2 fixation in water-stressed plants, indicating that maintenance of leaf-water potential should increase N_2-fixation potential during periods of water-stress. In this situation, breeding for drought-resistance and N_2-fixation may be more beneficial than selecting only for N_2-fixation potential, without regard to environmental adaptation.

Sinclair et al. (1991), using stability analyses in soybean, showed that those lines with the greatest mean nodule-weight also had the greatest positive response to favourable environments. Nodule weight and their number were highly correlated

traits (r>0.84). Root and shoot-weight had moderately positive correlatiions with nodule-number and nodule-weight. Heritability studies on nodulation indicate a potential for breeding to improve nodulation in soybean (Ronis et al., 1981; Wright, 1982; Greder et al., 1986). Seed yield in the study of Greder et al. (1986) was found to increase with an increase in the nodule-weight. Wright (1982) assessed the broad sense F_2-heritability estimates for nodule number, size and mass of 27 soybean genotypes. His estimates indicated that a substantial portion of the F_2 variance was due to genetic segregation. The distributions of F_1 and F_2 populations indicate the involvement of few genes that control host nodulation. In a recent study, Miller et al. (1991) have reported inheritance of non-nodulation in 10 sweetclover mutants, determined from F_1, F_2, and F_3 generations derived from crosses between each mutant and the nodulating accession. It was concluded that non-nodulation in each mutant was conditioned by a single recessive gene. The complementation analysis on F_1 and F_2 seedlings , derived from diallel crosses among the mutants, was also performed. It was found that five of the mutants were nonallelic, indicating that at least five different genes are involved in nodule-formation in sweetclover. In chickpea, a single recessive gene was found to control non-nodulation in all, except two mutants where no particular genetic pattern was observed (Singh and Rupela, 1997).

(ii) Estimatiion Methods for N_2 Fixation

The most widely-used methods of estimating N_2-fixation in legume crops include: 1) nitrogen accumulation; 2) difference methods; 3) isotope methods; and 4) techniques that assess variables associated with fixation (LaRue and Patterson, 1981; Weaver and Miller, 1986).

Estimation of total plant-N by standard procedures (such as Kjeldahl determination) is simple and inexpensive. Although these estimates may not reflect the precise amount of N_2 -fixation (since the N source cannot be determined accurately) they may provide reliable estimates for comparisons *between* lines. Even on poor-fertility soils, or when soil has been impoverished artificially, plants can scavenge the soil for N_2 and so estimates of

N_2-fixation based on total plant-N may be optimistic (LaRue-and Patterson, 1981).

The procedures used to account for the contribution of soil-N are known as difference methods. Alternative versions may employ comparisons of: 1) the test legume to a non-legume; 2) the test legume to a non-nodulating legume (as some of the non-nodulating mutants in sweetclover); 3) inoculated and uninoculated legumes; or 4) fertilizer equivalence (LaRue and Patterson, 1981). Comparisons of fixing legumes with a non-nodulating isoline of that crop are desirable, but isolines are limited to only a few standard cultivars of some legumes. Non-nodulating mutants have been described in different leguminous crops. A recent finding is reported in sweetclover (Miller et,al., 1991) and chickpea (Singh and Rupela, 1997). The non-nodulating variants can be appropriate non-fixing reference crops for quantification of N_2 fixed by nodulated chickpea by ^{15}N-based and difference methods (Rupela, 1997).

The difference method, which compares the N in un-inoculated and inoculated legumes, is feasible when there are no compatible native rhizobia. The sites having compatible native rhizobia in abundance may be used for idenitfyng plant-genotype capable of being nodulated, selectively, by a superior strain in competition with wild rhizobia.

The N accumulated by the nodulated legume may be compared to that in similar legume-plants by giving different amounts of fertilizer N. The comparisons of plant-yields, from addition of fertilizer N with those of plants fixing N_2, may help to identify plant-genotypes capable not only of fixing N_2 in the absence (or presence) of combined N, but also responding to different amounts of added N.

N_2 fixation can be estimated by ^{15}N-isotope dilution, where the fixing legume and a non-fixing control are grown on soil to which ^{15}N has been added as labelled nitrate or ammonium (McAuliffe et al., 1958). With this method, there are certain disadvantages like non-uniformity of enrichment in the soil and the ability to obtain only

semi-quantitative data. A thorough review of procedures and potential sources of error should precede use of isotope methods (Hauck and Bremner, 1976).

Fixation and fixation-potential have been estimated indirectly from variables associated with fixation. The acetylene-reduction assay (ARA), based on the fact that nitrogenase reduces acetylene to ethylene, has been widely used. Many versions of the method exist (Hardy et al., 1973); the most common involves incubation of freshly-excised nodulated roots in a vessel with 1-20% C_2H_2 for 30-120 min, after which a sample of the gas- phase is removed and the ethylene produced measured by a gas chromatograph (LaRue and Patterson, 1981). Conventional use of ARA involves destruction of the plant, making repeated observations impossible. The ARA method has been used in breeding studies to obtain estimates of differences among genotypes for fixation-potential (McFerson, 1983). These estimates were most useful when replicated samples were collected together with data such as plant biomass and seed-yield for the same experimental lines.

Other indirect methods, like nodule number and mass per plant, leghaemoglobin concentration in nodules, visual nodulation scores, and ureide concentration in the xylem sap or in various plant parts (Rosas and Bliss, 1986), have been used to estimate fixation -potential. These methods provide indicators of nodulation and active fixation, and can thus be used with appropriate caution to provide rankings among genotypes being evaluated for selection.

(iii) Molecular Approach

In recent years, considerable attention has been given to nodule-specific proteins, i.e. proteins or polypeptides found only in nodules and not in the subtending roots or other plant-parts. While such proteins may also be of bacterial origin, attention has been focused on those nodulins derived' from the host, in the belief they will provide information on factors involved in nodule formation or nodule function. The most obvious nodulins are the leghaemoglobin proteins (Gibson, 1988). Research

has shown that the production of these proteins is under the control of four very similar leghaemoglobin genes located in two clusters (Lba, Lbc1, Lbc3 and a pseudogene; Lbc2 and the second pseudogene) each flanked by regions that cross hybridize (Verma et al., 1984).

Transcription of at least three of these genes commences before the nodules can be observed. A chimeric soybean Lbc3 gene, containing the chloramphenicol acetyl transferase coding sequence, was introduced into *Lotus corniculatus* root-tissue, using *Aqrobacterium rhizogtenes* Ri plasmid (Gibson,, 1988) . Plants derived from this tissue culture were inoculated with *Rhizobium loti* and evidence was obtained for expression of the soybean Lbc3 gene, although only in the nodule tissue (Jensen et al., 1986) . It shows that Lb genes can be transferred and expressed in other legumes.

Over 30 nodulins have been found in developing nodules. Nodulins were identified using extraction of mRNA, which is reverse-transcribed to produce cDNA. The cDNA is used to provide a library of clones, which are further selected on the basis of specificity of reaction with nodule-mRNA but not root mRNA. These probes have been used to study the temporal development of nodulins in nodules formed by effective strains of rhizobia, and by various ineffective mutants on different legume species (Gibson, 1988).

Traits that have been altered, using the molecular tools of genetic engineering, are improvements in competition, enhancement of nodulation, alteration in surface polysaccharide production and host range, energy utilization, bacteriocin production, and nitrogen fixation (Thomas et al., 1997). Genes presumed to be involved in competitiveness have been isolated from *B. japonicum* USDA438 by subtractive DNA hybridization from *B. japonicum* USDA110 (Bhagwat and Keister 1992). Transfer of genes from *B. japonicum* USDA438 to less competent strain, USDA110, increased the competitive nodulation ability of the strain. At the same time, production of antirhizobial compounds, trifolitoxin, by rhizobial inoculant strains could be a useful strategy in limiting nodulation by indigenous strains. The trifolitoxin genes have

been cloned and transferred to effective strains of Rhizobium by conjugation of a recombinant plasmid, pTFX1, which possesses the genes for trifolitoxin production and resistence (Triplett, 1988; Triplett, 1990).

(iv) Plant Breeding Methods

The choice of breeding methods is influenced by: 1) the complex nature of the expression of N_2-fixation; 2) trait-heritability; and 3) the destructive nature of many methods for estimating N_2-fixation. The complex nature and moderate to poor heritability of N_2 fixation and related variables suggest that replicated evaluations will help to identify superior genotypes. Replicated family testing also allows for testing against native rhizobia or single inoculant strains, for interactions with added N and, perhaps most important, the evaluation of each family or other important traits, like earliness, adaptation, disease-.reaction and yield (Bliss and Miller, 1988).

The evaluation of families (lines) that can be replicated rather than individual plants, as the units of selection is more efficient (Bliss and Miller, 1988) . On the basis of this consideration, breeding methods that appear to be well-suited for improving N_2 fixation in the legumes are the modified pedigree method (Brim, 1966) and the inbred backcross line method (McFerson et al., 1983). The superior families, selected on replicated family mean-performance, can be released as new cultivars, or used as parents for further crossing.

Nodulation variants, within released cultivars of groundnut, were selected to identify high-nodulating lines to select plants with high nodule number and biomass at harvest, and for growing single-plant progenies in the next year using the pure line selection method. High-nodulating lines consistently maintained superior nodule biomass and gave significantly higher pod-yield than the control, indicating the promise of this technique to obtain high-nodulating lines and yield (Venkateswarlu, 1997).

FUTURE PROSPECTS

Legume host-plant breeding for increased N_2-

fixation

seems to be feasible. Host-plant improvement provides both direct and indirect benefits; plants are able to nodulate more profusely and to support larger N_2 fixation values; _Rhizobium_ superior strains may also be distinguished more easily using a superior host. Further evaluation of legume germplasm for genotypes possessing traits that affect N_2 fixation is needed. Some plant characteristics may limit N_2 fixation. Early maturing determinate plants seem to fix less N_2, than late indeterminate plants with greater leaf-canopy. Significant increases in fixation may be achieved in each plant-type through host-plant improvement, either in relation to an individual superior rhizobial strain or a mixed native population.

Some plant characteristics may not allow large rates of N_2 fixation to be transferred into increased seed yield. Weaver and Miller (1986) reported good and poor fixing mungbean cultivars, which differed substantially in their ability to remobilize N from vegetative tissue to fruits during reproductive growth. If efforts to increase seed-yield by increasing N_2-fixation are to be successful then the genotypes developed should also .be able to partition the increased N into the seeds of legume genotypes.

Comparative yields of plants receiving N-fertilizer are often greater than those fixing N_2 (Bliss and Miller, 1988). Effective breeding for increased N_2-fixation may produce plants as productive as those receiving N. At the same time, there are some evidences that plants and rhizobia differ in their response to the presence of large amounts of external N. This may give an opportunity to develop plants capable of fixing substantial N_2, in the presence of either large or small. amounts of external N, leading to increased productivity.

Work at molecular level in identifying the genes involved in the symbiosis and the incorporation of these genes into either partner may speed up the work to achieve the task of enhancing N_2-fixation in the future.

Development of more competitive rhizobial strains, regular training of extension staff and farmers, using appropriate training- materials, well-planned

on-farm demonstrations, and the establishment of Rhizobium inoculum production-centres at provincial levels, are suggested to enhance benefits from this low-cost and environment-friendly technology.

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