# COWPEA

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# *Selection for Enhanced Nitrogen Fixation in Cowpea*

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Biological nitrogen fixation in cowpea, as in other legumes, is a process whereby *Rhizobium* spp., in association with the host plant, reduce free nitrogen from the soil air to a form usable by the plant. The role played by the plant in the symbiosis is to furnish carbohydrates that supply energy to the nitrogen-fixing bacteria, while nitrogen compounds produced by the bacteria are used by the plant for growth. Obviously only a small, but sometimes significant, portion of the carbohydrates produced by the host plant is used to supply the energy requirements of the bacteria. Likewise, not all nitrogen fixed by the bacteria is made available to the host plant for its use. Nitrogen may pass into the soil by excretion, by the sloughing off of the roots, especially their nodules, or by the decomposition of the cowpea plant at the end of the season. Thus, crops grown in association with cowpea or succeeding crops may benefit from the fixed nitrogen.

There is general agreement that cowpea yields could be increased through proper plant nutrition, especially nitrogen, if other factors such as disease and insect incidence were controlled through plant resistance or other means. There is, however, disagreement as to precisely how much of the cowpea plant's nitrogen requirement could be met through biological nitrogen fixation alone. Many factors, such as plant genotype, quality and quantity of available rhizobia and numerous environmental factors such as available soil nitrogen, extreme temperatures, low pH, lack or excess of soil moisture, etc., influence the biological nitrogen fixation process. The important point is that biological nitrogen fixation could contribute more to cowpea yield than it is at present if it were being fully exploited. For example, we found that, on low nitrogen soils (<15 kg/ha), the benefits in seed yields derived from rhizobial

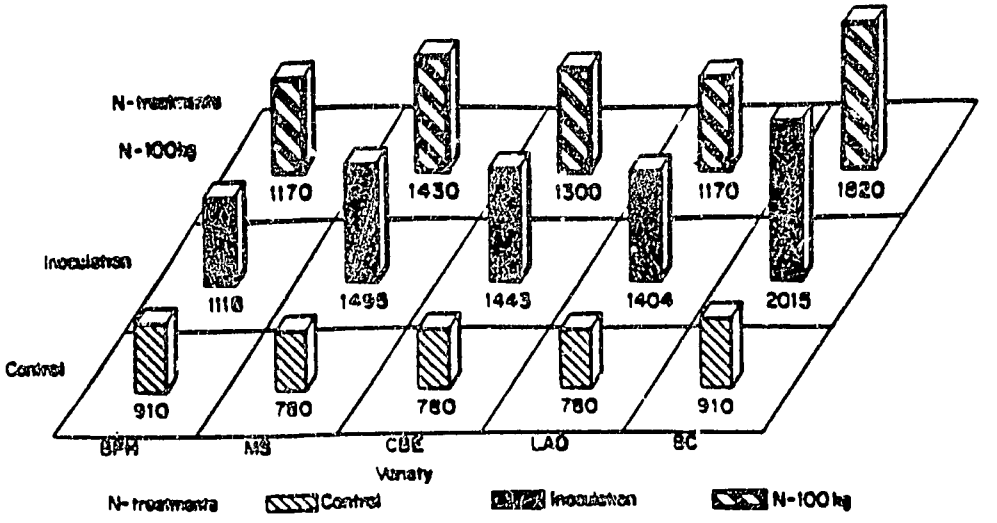


Figure 1. Effects of inoculation vs 100 kg/ha nitrogen on seed yield (kg/ha) in cowpea varieties 'Bush Purple Hull' (BPH), 'Mississippi Silver' (MS), 'California Black Eye No. 5' (CBC), 'Lady' (LAD) and 'Brown Crowder' (BC)

inoculation compared with noninoculation were equivalent to at least 100 kg/ha of fertilizer nitrogen (Figure 1).

## THE NITROGEN FIXATION SYMBIOSIS

Several steps need to be successfully completed before effective biological nitrogen fixation can occur. The process begins on the root surface and follows a multistage sequence of interdependent steps culminating in the establishment of an effective $N_2$-fixing nodule. Briefly stated, this series of events starts with the bacteria contacting the surface of the root hair, perhaps with lectin-assisted recognition, curling of the root hair, and penetration of the root by the bacteria. After entering, the bacteria form infection threads through which they migrate to the root cortex, the first layer of cells under the epidermis. Nodules are then formed as the result of plant-cell division and cell enlargement provoked by the bacteria. The bacteria then increase in the nodule tissue and develop bacteroids, which have the capacity to fix nitrogen.

To breed for increased biological nitrogen fixation in cowpea, or any other legume species for that matter, one needs to have some understanding of the nature of the symbiosis. The events in the symbiotic process can be grouped in a number of ways to conform with three major steps that are commonly agreed upon, namely: preinfection, infection and nodule development and nodule function (Vincent, 1980) (Table 1). This sequence of events is

Table 1. Analysis of symbiotic sequence in biological nitrogen fixation (Vincent, 1980)

| Stage | Abridged description | Phenotypic code |
|---|---|---|
| Preinfection | | |
| Multiplication on root surface ('rhizoplane') | Root colonization | Roc |
| Attachment to root surface | Root adhesion | Roa |
| Branching of root hairs | Hair branching | Hab |
| 'Marked' curling of root hairs | Hair curling | Hac |
| Infection and nodule formation | | |
| Formation of infection thread | Infection | Inf |
| Development of polyploid (disomatic) meristem, nodule development and differentiation | Nodule initiation | Noi |
| 'Intracellular' release of rhizobia from infection thread | Bacterial release | Bar |
| 'Intracellular' multiplication of rhizobia and development of full bacteroid form | Bacteroid development | Bad |
| Nodule function | | |
| Reduction of $N_2$ to $NH_4^+$ (nitrogenase) | Nitrogen fixation | Nif |
| Complementary biochemical and physiologic functions | Complementary functions | Cof |
| Persistence of nodule function | Nodule persistence | Nop |

complex as well as interrelated and interdependent. Furthermore, attempts by plant breeders to define the genetic, physiologic or environmental control of symbiosis in such general terms as nodule number (invasion, nodule initiation or nodulation) and nitrogenase activity (nodule effectiveness) leave them open to criticism by microbiologists.

Breeders need to recognize that looking at components such as nodule number is examining only one of several components that contribute to infection and nodule formation. Likewise, when looking at nitrogenase activity, they are dealing with only one facet of the broader component of nodule function. At the same time the microbiologist must recognize the difficulty in breeding for (i.e. evaluating, indexing) a trait that cannot be seen

without a microscope. Also the way to improve the whole is by improving its component parts, and the parts that are easiest to deal with from a breeding standpoint will probably be the first order of business for the breeder.

## IMPROVING THE SYMBIOSIS

Interactions between the host plant and *Rhizobium* are complex, and made even more complicated by the influence of environment. Little can be accomplished by debating whether host plant, microbial symbiont or environment is most important, as cooperation among all three is essential. In fact, for symbiosis at maximal levels of efficiency, all three factors must mesh perfectly.

### Rhizobial approach to enhancing biological nitrogen fixation

Historically, the study and enhancement of biological nitrogen fixation have centred on the microsymbiont, with efforts to increase nitrogen fixation efficiency channelled primarily toward the development of improved strains of *Rhizobium*. These improved strains have not always had an impact on cowpea production. The introduction of new strains into soil where indigenous strains are well established is frequently unproductive. The introduced strains are usually not as competitive as the indigenous strains for survival in that soil and to effect good nodulation must often be introduced at uneconomic levels (LaRue, 1980).

It is frequently proposed that superior or improved strains of *Rhizobium* be provided to farmers—an approach that can and does work under certain circumstances. However, the cost of implementing such a proposal can be high, when the establishment of an inoculant factory and distribution of the inoculant to farmers are considered. Additional obstacles to this approach include the fact that rhizobia are quite sensitive to desiccation and heat, and developing countries lacking an adequate transportation network may find distribution of viable inoculant difficult or impossible. An alternative approach is the development of cowpea cultivars capable of enhanced, effective symbiosis with indigenous rhizobia.

### Host plant genetics and enhancing biological nitrogen fixation

Historically, those attempting to increase fixation have concentrated on the *Rhizobium*, ignoring the contribution of the host plant. The most notable exception is P. S. Nutman (now retired) of the Rothamsted Experimental Station who, for almost 40 years, investigated the plant genetics of nodule formation in clover and expounded on the need to study the host plant as well as the microsymbiont.

The host plant is involved in determining when and which strains infect the root, the extent of nodulation, and the potential for $N_2$ fixation (Graham, 1982). In fact, the host can control all steps of the symbiotic sequence (Table 1). It is only speculative and probably unproductive to suggest the number of host genes that contribute to such a complex process, but many must be involved. The identification and manipulation of this plant genetic material is essential if the potential contribution of biological nitrogen fixation is to be realized.

Plant breeders have rarely consciously selected for biological nitrogen fixation, and the $N_2$-fixing potential of new lines has seldom been evaluated and reported. Only in the past 10 years have they begun to recognize fully the contribution of the host genotype and the potential for its exploitation in maximizing the benefits from symbiosis. However, the research community working on biological fixation now includes a small number of plant physiologists and plant breeders in addition to the microbiology core. There are now a number of programmes throughout the world that are attempting to develop legume cultivars with improved nitrogen-fixation potential.

## ENHANCING BIOLOGICAL NITROGEN FIXATION IN COWPEA

The Texas A&M University programme to improve nitrogen fixation in cowpea was established in 1976. The programme has an active microbiology component; however, most of the work has centred on plant physiology and genetics as they relate to nitrogen fixation in cowpea. The programme was initiated with the development of a screening procedure for detecting quantitative differences in nitrogenase activity, nodule number, nodule weight and plant top weight, while keeping rhizobial strains constant. To date, more than 1400 genotypes have been screened, and extremely wide variability for all traits has been found (Zary *et al.*, 1978). However, non-nodulating cowpea genotypes have yet to be identified with this procedure.

Numerous studies have been conducted, including the effect of viruses, mycorrhizal fungi, soil nitrogen (including nitrogen uptake and partition), soil phosphorus, plant density (population), water and temperature stress, and grafting to determine the effect of root stock and scion on nitrogen fixation in cowpea (Zary and Miller, 1980; O'Hair and Miller, 1982). In addition, rather extensive investigations on the genetics of the host plant have been, and are currently being, carried out. For example, investigations on the genetics of quantitative differences in nodule number (nodulation or nodule initiation) and nitrogenase activity (nodule function) resulted in heritability estimates of 0.55 and 0.62, respectively, indicating that progress should be significant in breeding for enhanced biological nitrogen fixation in cowpea by improving these two components (Tables 2 and 3).

Table 2. Variance components and heritability estimation on log-transformed data in a cross between cowpea varieties Brown Crowder ($P_1$) and Bush Purple Hull ($P_2$) for nodules/plant*

| Generation | N | $\sigma^2$ | Three-parameter model | | |
|---|---|---|---|---|---|
| | | | Observed means | Expected means | Residual |
| $P_1$ | 20 | 0.0169 | 1.292 | 1.313 | −0.021 |
| $P_2$ | 20 | 0.0096 | 1.590 | 1.594 | −0.004 |
| $F_1$ | 20 | 0.0118 | 1.520 | 1.549 | −0.029 |
| $F_{1'}$ | 20 | 0.0135 | 1.560 | 1.549 | 0.010 |
| $BC_1$ | 80 | 0.0202 | 1.483 | 1.431 | 0.052 |
| $BC_2$ | 80 | 0.0234 | 1.592 | 1.571 | 0.021 |
| $F_2$ | 240 | 0.0300 | 1.501 | 1.501 | 0.000 |

* Variance components include additive variance 0.0329, dominance variance 0.00239, environmental variance 0.0129, degree of dominance 0.27 and narrow sense heritability 0.55. The chi-square value is 0.34, indicating that the three-parameter model is adequate.

Table 3. Variance components and heritability estimation on log-transformed data in a cross between cowpea varieties Brown Crowder ($P_1$) and Bush Purple Hull ($P_2$) for nitrogenase activity*

| Generation | N | $\sigma^2$ | Three-parameter model | | |
|---|---|---|---|---|---|
| | | | Observed means | Expected means | Residual |
| $P_1$ | 20 | 0.0139 | 0.744 | 0.735 | −0.009 |
| $P_2$ | 20 | 0.0196 | 0.285 | 0.302 | −0.017 |
| $F_1$ | 20 | 0.0190 | 0.648 | 0.641 | −0.007 |
| $F_{1'}$ | 20 | 0.0209 | 0.629 | 0.641 | 0.012 |
| $BC_1$ | 80 | 0.0387 | 0.627 | 0.688 | −0.061 |
| $BC_2$ | 80 | 0.0429 | 0.542 | 0.472 | 0.070 |
| $F_2$ | 240 | 0.0593 | 0.589 | 0.580 | 0.009 |

* Variance components include additive variance 0.0738, dominance variance 0.0162, environmental variance 0.0183, degree of dominance 0.47 and narrow sense heritability 0.62. The chi-square value is 0.32, indicating that the three-parameter model is adequate.

Cowpea is probably one of the best candidates for plant breeding attention in the area of nitrogen fixation. It could certainly be classified as a poor person's crop, worldwide and, as such, the likelihood that it will be grown under optimal nutritional conditions, especially with expensive fertilizer nitrogen, is negligible. Therefore, attention should be directed and focused on meeting nutritional needs for maximal seed and biomass yield, through full exploitation of biological nitrogen fixation. As it is difficult or impossible to control the environment, attention should be directed to the microbial symbiont and the host plant. The most promising approach would be that of developing host plants with superior biological nitrogen fixation characteristics.

With the identification of biological nitrogen fixation components that can be seen and worked with, variability for these factors indexed and found to be substantial, and encouraging heritability estimates, a breeding strategy was then formulated. However, at least two important and rather unique considerations had to be kept in mind in establishing the methodology. First, superior levels of biological nitrogen fixation cannot be seen by the breeder through casual observation and, secondly, measurement of relative levels of fixation usually requires destruction of the cowpea plant, obviously leading to a non-productive dead end. These problems can be overcome if breeders take cuttings before, or in conjunction with, whatever procedure is used to measure a given nitrogen fixation component. Dealing with large numbers of plants makes this approach cumbersome, time-consuming and, consequently, expensive.

With these limitations and restraints in mind we propose two breeding approaches. The first, and by far the easiest and at the same time least likely to meet with success, simply involves the selection of parents from genotypes that have been identified as possessing high nitrogen-fixation potential. In this case, whether the pedigree, population improvement, or any other method is followed, no monitoring of progeny for nitrogen-fixing potential throughout successive generations is practised. Breeders would depend on the relatively high heritabilities for the various nitrogen-fixation components to ensure that these desirable traits are passed forward. For example, they might follow this approach in a programme for disease resistance. In selecting parents, breeders might choose lines that, in addition to possessing resistance to the disease, also possess high nitrogen-fixation potential.

The second method is a modification of that proposed for common bean by McFerson *et al.* (1982) and involves a continual assessment that is not directly destructive and does not involve cuttings. We describe this approach as a modified backcross–inbred method and believe that it offers a high probability for success. Cowpea lines possessing high $N_2$-fixation potential (donor parent) are crossed to an adapted, high-yielding cultivar (recurrent parent),

and approximately 30 $F_1$ hybrid seeds are obtained (Figure 2). These seeds are grown in the greenhouse and crossed to the recurrent parent and approximately 100 backcross (BC) seeds are obtained. Each BC seed is a different genotype, as the inheritance of $N_2$ fixation is quantitative. Therefore, the selfed offspring from each BC seed constitutes a unique family.

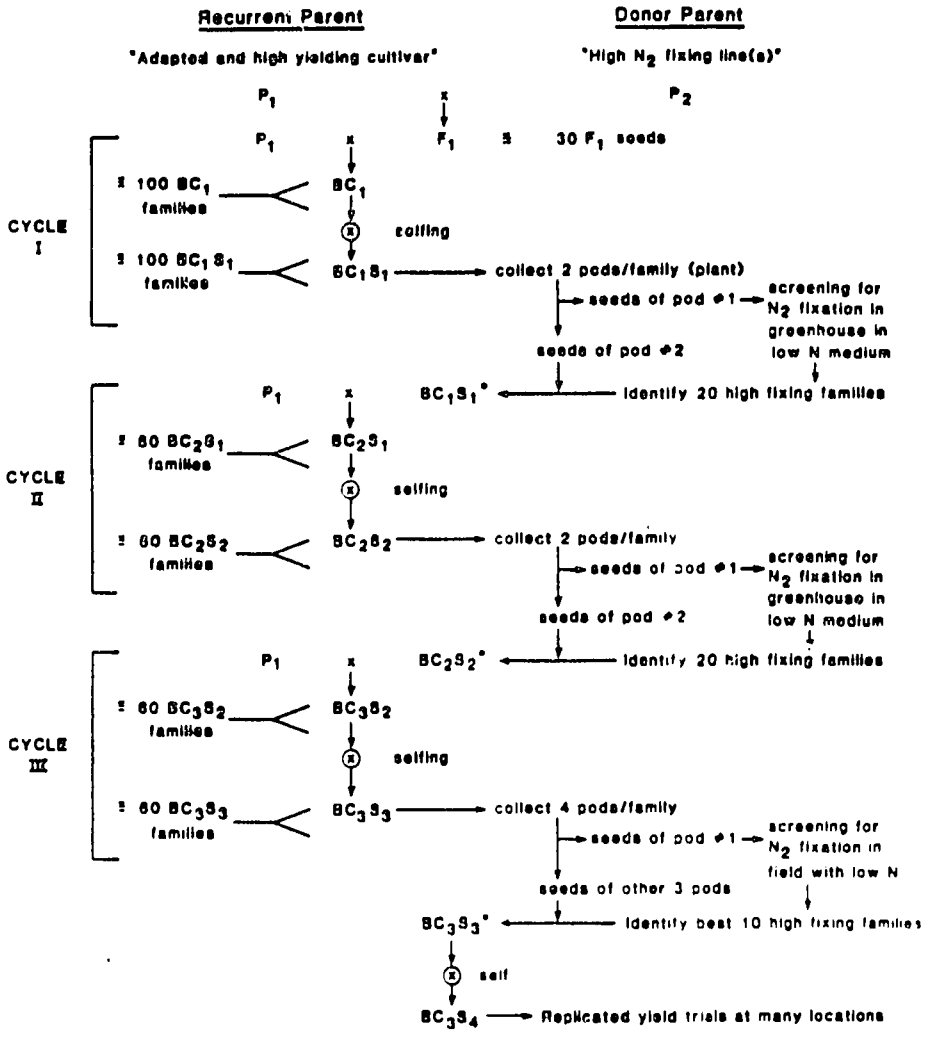


Figure 2. Modified backcross–inbred method to introduce high $N_2$ fixing potential into an adapted and high-yielding cultivar

Seeds of the first backcross ($BC_1$) are grown and selfed (S) in the greenhouse. From each $BC_1$ family, two pods are collected and the selfed progeny identified as $BC_1$ $S_1$. Seeds from one pod are grown in a nitrogen-free medium in the greenhouse, and the $BC_1$ families evaluated for $N_2$-fixation potential based on the mean performance of the selfed progeny. Seeds of 20 high $N_2$-fixing families ($BC_1$ $S_1$) from the second pod are grown in the greenhouse and advanced to the second cycle. Thus, one cycle involves a backcross, selfing, evaluation for $N_2$ fixation, and identification of high-fixing families.

This cycle is repeated twice, and in cycle II, about 80 $BC_2$ $S_2$ families are evaluated and 20 $BC_2$ $S_2$* high fixing families identified. In cycle III, 60 $BC_3$ $S_3$ families are evaluated in the field, with low nitrogen. Four pods are collected from each family and one pod used for evaluation, while the other three pods are used for seed multiplication. The best 10 high-fixing families ($BC_3$ $S_3$*) are selected and seeds multiplied in the field by selfing. The $BC_3$ $S_4$* families are evaluated for yield and $N_2$-fixation potential in many locations. This method comprises three alternating cycles of BC and S, then one final selfing. Therefore, at the end of this breeding procedure, the selected plants will have achieved greater than 98 per cent homozygosity. This modified backcross–inbred method offers several advantages for the introduction of superior $N_2$-fixation potential into an adapted cultivar:

- Only the high $N_2$-fixing families are carried forward to the final selection; therefore the probability of success is high.
- Evaluation of a BC family from the mean performance of the selfed progenies facilitates screening for quantitative traits such as $N_2$-fixation potential.
- The tedium and expense of taking cuttings and growing them out are eliminated.
- The destructive $C_2H_2$–$C_2H_4$ assay is not a problem since screening is carried out on the selfed progenies from seed of an extra pod harvested for this purpose.
- The number of crosses required is reduced because with three BC and four S more than 98 per cent homozygosity is attained.

## CONCLUSION

In conclusion, biological nitrogen fixation in cowpea depends on a complex interaction between host plant, microbial symbiont and environment. Microbial symbiont as well as host-determined factors influence nodule initiation, development and function. Both partners of the symbiosis are subject to genetic variation; thus, substantial variation exists in type of nodulation, amount of nitrogen fixed and, ultimately, cowpea yield. Evidence is that the genetic manipulation of the host plant offers the greatest potential for improvement of current levels of biological nitrogen fixation in cowpea.