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# The Influence of Available Nitrate Levels on Nitrogen Fixation in Three Cultivars of Cowpea[1]

J. C. Miller, Jr.[2], J. S. Scott[3], K. W. Zary[3], and S. K. O'Hair[3]

## ABSTRACT

**Soil N is known to affect $N_2$ fixation in legumes Increased cowpea yields might be achieved by increasing N assimilation through greater symbiotic fixation, but identification of high-fixing genotypes requires an effective screening technique. The purpose of this investigation was to determine the optimum level of supplemental N for maximum $N_2$ fixation in cowpeas grown under greenhouse and field conditions, and the relative contributions of the measured variables on nitrogenase activity.**

**Three cowpea cultivars were tested for $N_2$ fixation, $N_2$ ($C_2H_2$), under greenhouse conditions with 0, 5, 10, and 20 ppm N applied as $Ca(NO_3)_2$ and under field conditions with 0, 11.2, 22.4, and 44.8 kg/ha N applied as $Ca(NO_3)_2$. The soil was Vertic Albaqualf, fine, Montmorillonitic thermic. Nitrogenase activity decreased with increasing rates of N. Path analysis indicated that nitrate effects on $N_2$ fixation activity were primarily on nodule mass, with the inhibition of nodule growth rather than nodule initiation. Nodule number was not a reliable predictor of $N_2$ fixation potential of cowpea genotypes and was negatively correlated with nitrogenase activity. The results clearly indicated no advantage of applied nitrate in promoting maximum genotypic differences for $N_2$ fixation.**

*Additional index words: Vigna unguiculata, Vigna sinensis*, Southernpea, Nitrogenase, Acetylene reduction, Nodulation, Nitrate inhibition.

COWPEAS [*Vigna unguiculata* (L.) Walp] and other legumes do not respond by yield increases to soil or applied N to the same degree as other plant families. A possible solution to increased N assimilation and subsequent enhanced yields is through maximizing fixation of atmospheric N. The three broad areas for improvement of the symbiosis include: improved plant genotypes, improved inoculant, and optimization of environmental conditions. These three components are interrelated. Recently, increasing emphasis has been placed on identifying and developing cowpea genotypes with increased genetic potential for $N_2$ fixation (10). In order to pursue this approach, the optimum environmental conditions for fixation must be determined and provided so that genotypic extremes can be identified more accurately.

Available soil N has a rather strong influence on $N_2$ fixation. Decreased $N_2$ fixation by legumes has long been recognized as a detrimental effect of nitrates. Previous research on cowpea has shown that high levels of soil or media N inhibit $N_2$ fixation, while low levels of N may promote fixation (2, 3, 4, 8). The interactions among temperature, light intensity, and ammonium nitrate on growth and nodulation of cowpeas in the growth chamber have been reported (2). In a greenhouse study of *Rhizobium* strain interactions with applied N, it was found that fixed N increased from rates of N in the range of 0 to 160 mg N (200 ppm) per pot, then decreased at 240 mg (300 ppm) N (3). In another strain study conducted in the greenhouse where 50, 100, 150, and 200 ppm N as $KNO_3$ were applied to cowpeas inoculated and grown in sterile sand, the N content of the plants was increased by addition of N up to 150 ppm, then declined at 200 ppm N (4). In a greenhouse study on the effects of environmental stress on $N_2$ fixation and seed yield of cowpeas, nitrogenase activity for plants receiving no nitrate was less than 1/10 of that for plants receiving 25 ppm N (8). The N treatments had little if any effect on final seed yield for nodulated plants.

The objectives of this investigation were to determine: (i) the optimum level of supplemental N for maximum $N_2$ fixation activity in cowpeas grown under greenhouse and field conditions and (ii) the relative contributions of the measured variables on nitrogenase activity, in order to establish parameters for screening cowpea genotypes in a breeding program.

## MATERIALS AND METHODS

*Greenhouse Experiment.* A split-plot design with three blocks was utilized, with main plots consisting of nitrate treatments 0, 5, 10, and 20 ppm N as $Ca(NO_3)_2$ added to a N-free nutrient solution (7). Subplots consisted of three cowpea genotypes previously identified as having high or low $N_2$ fixation potential (10). These cultivars included: 'Chinese Red' (low), 'Knuckle Purple Hull' (high), and 'California Blackeye No. 5' (high). Variables measured included nitrogenase activity, nodule fresh weight, nodule number, and dry weight of the plant tops.

Seeds were inoculated with a commercial, mixed strain, granular cowpea *Rhizobium* inoculant and grown in 3.8-liter plastic pots containing a 1:1 mix of sand and coarse vermiculite. Plants were grown for 6 weeks, then tested for $N_2$ fixation activity by the acetylene-ethylene assay. Plants were severed at the soil line and tops placed in individual bags for drying. Roots, with nodules intact, were carefully removed from the potting medium and immediately placed into 0.48-liter septum-fitted, gas-tight Ball jars. These jars were then injected with 25 cc of $C_2H_2$ (5%). After a 1-hour incubation at 25 C, 3 cc of the gas mixture was removed and injected into gas-tight vacutainers for later analysis. Ethylene was determined by gas chromatography and hydrogen flame ionization. The root-nodule complex was then removed from the jars, placed into individual labelled plastic bags to prevent dehydration and frozen for later determination of nodule number and mass.

Data were analyzed by correlation and path coefficient analysis, because measured variables were not independent. Multiple linear regression was used to obtain the standard partial regression coefficients and correlation coefficients for each cultivar separately and all three combined.

*Field Study.* A split-plot design with four blocks was used, with main plots consisting of nitrate treatments of 0, 11.2, 22.4, and 44.8 kg/ha N as $Ca(NO_3)_2$. Subplots consisted of the same cultivars used in the greenhouse experiment. Variables measured included: nitrogenase activity, nodule fresh weight, nodule number, dry weight of shoots, shoot protein, and soil nitrate levels. The experiment was conducted at College Station, Tex. on a Vertic Albaqualf, fine Montmorillonitic, thermal soil at pH 6.7. Calcium nitrate

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was applied to the plots by hand and incorporated to a depth of 12 cm before planting on 7 June 1977. Seeds were treated with a commercial, mixed strain, granular cowpea *Rhizobium* inoculant and planted on 100 cm rows. After emergence, plants were thinned to 20 cm within rows and watered as needed by sprinkler irrigation. Nitrogen fixation activity was determined by the acetylene-ethylene assay, at the mid-pod-fill growth stage on 26 July 1977, 7 weeks after planting. Four plants of each cultivar were harvested from the center row of each three-row main plot.

Soil samples from each main plot were collected before fertilizer application on the day of planting, and again at 21, 40, and 49 days after planting (date of harvesting). Samples were frozen until the end of the experiment, when nitrate levels were determined by colorimetric analysis (1).

Protein content of the shoots was determined after 7 weeks of growth, by bulking sampled plants of each cultivar by nitrate treatment and block. Large stems were removed and samples of the leaves and petioles were ground. Total N content was determined by micro-Kjeldahl technique.

## RESULTS

*Greenhouse.* Path coefficient analysis was used to partition effects of each measured variable on nitrogenase activity because measured variables were not independent. Nodule fresh weight may affect nitrogenase activity directly, but it may in turn be affected by nodule number, shoot dry weight, and nitrate. While these possible interactions may be easily recognized, their quantitative effects on nitrogenase activity may not be obvious. Correlation and path analysis allows for the partitioning of the various effects of each measured variable into direct effects on nitrogenase activity and indirect effects through other variables, which in turn affect nitrogenase activity. Thus, the relative effects of each measured variable on nitrogenase activity, as well as the relationships among the variables measured, can be determined. The relationships among the variables studied are presented diagrammatically in Fig. 1.

Multiple linear regression was performed to obtain simple correlation coefficients and standard partial regression coefficients. Linear regression equations are presented in Table 1. Path coefficients were obtained by simultaneous solution of the following equations in which '$r$' values are simple correlation coefficients and '$P$' values are standard partial regression coefficients (6):

$$r_{15} = P_{15} + r_{12}P_{25} + r_{13}P_{35} + r_{14}P_{45}$$
$$r_{25} = P_{25} + r_{12}P_{15} + r_{23}P_{35} + r_{24}P_{45}$$
$$r_{35} = P_{35} + r_{13}P_{15} + r_{23}P_{25} + r_{34}P_{45}$$
$$r_{45} = P_{45} + r_{14}P_{15} + r_{24}P_{25} + r_{34}P_{45}$$

Analysis of correlation coefficients (Table 2) elucidates the nature of the relationships among the vari-

**Table 1. Linear regression equations for $N_2$ fixation variables measured in greenhouse and field experiments.**

| Cultivar | Regression equation† |
|---|---|
| | Greenhouse experiment |
| Chinese Red | $Y = -0.0630 - 0.0518 X_1 + 0.0281 X_2 - 0.0280 X_3 + 0.0004 X_4$ |
| Knuckle Purple Hull | $Y = 3.7127 - 0.0713 X_1 + 0.0143 X_2 - 0.0562 X_3 - 0.0002 X_4$ |
| California Blackeye No. 5 | $Y = 2.7005 + 0.0421 X_1 + 0.0135 X_2 - 0.0088 X_3 - 0.0018 X_4$ |
| Pooled cultivars | $Y = 2.5183 - 0.0442 X_1 + 0.0145 X_2 - 0.0471 X_3 + 0.0001 X_4$ |
| | Field experiment |
| Chinese Red | $Y = 7.5158 - 0.0648 X_1 + 0.0137 X_2 - 0.0425 X_3 + 0.0291 X_4 - 0.2401 X_5$ |
| Knuckle Purple Hull | $Y = 4.2169 + 0.0677 X_1 + 0.0122 X_2 + 0.0365 X_3 - 0.0152 X_4 - 0.3712 X_5$ |
| California Blackeye No. 5 | $Y = 57.4441 - 0.0096 X_1 + 0.0170 X_2 - 0.0504 X_3 - 0.0660 X_4 - 1.9434 X_5$ |
| Pooled cultivars | $Y = 12.4783 - 0.0287 X_1 + 0.0148 X_2 - 0.0122 X_3 - 0.0242 X_4 - 0.4600 X_5$ |

† Y = Nitrogenase activity (μmoles $C_2H_4$/plant•hour); $X_1$ = ppm N as $Ca(NO_3)_2$ in GH, $X_1$ = Nitrate (kg/ha) in field; $X_2$ = Nodule fresh weight/plant, (mg); $X_3$ = Nodule number/plant; $X_4$ = Shoot dry weight (g); $X_5$ = Shoot protein (% of dry weight).

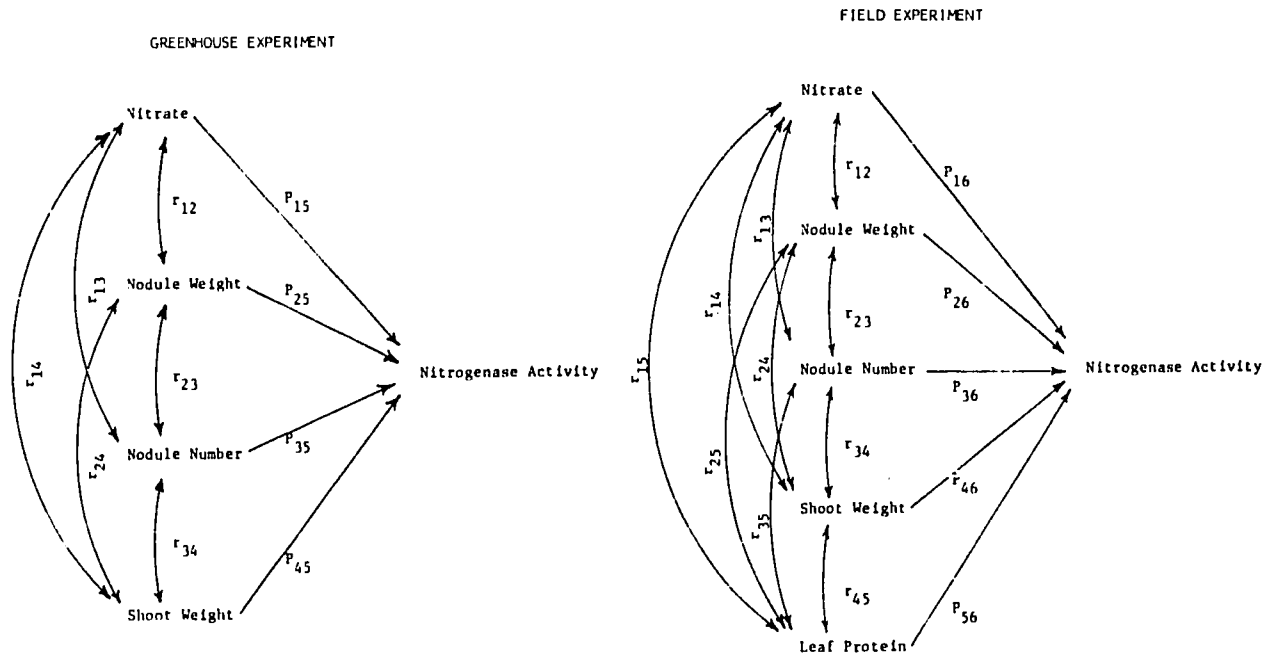


**Fig. 1. Path analysis diagram on $N_2$ fixation variables studied, as affected by nitrate treatment, for greenhouse and field experiments.**

**Table 2. Correlation coefficients by path analysis of relationships among $N_2$ fixation variables studied in greenhouse and field experiments, using pooled cultivars.†**

| Variables | | Greenhouse | Field |
|---|---|---|---|
| Nitrate vs. Nitrogenase activity | r = | −0.3527 | −0.3029 |
| Direct effect | | −0.1220 | 0.0230 |
| Indirect effect via nodule wt. | | −0.2434 | −0.3683 |
| Indirect effect via nodule no. | | 0.0116 | 0.0133 |
| Indirect effect via shoot wt. | | 0.0008 | −0.0024 |
| Indirect effect via shoot protein | | | 0.0286 |
| Total | | −0.3530 | −0.3028 |
| Nodule wt. vs. Nitrogenase activity | r = | 0.6267 | 0.7947 |
| Direct effect | | 0.8730 | 0.8610 |
| Indirect effect via nitrate | | 0.0340 | −0.0111 |
| Indirect effect via nodule no. | | −0.2890 | −0.0395 |
| Indirect effect via shoot wt. | | 0.0094 | −0.0043 |
| Indirect effect via shoot protein | | | −0.0112 |
| Total | | 0.6274 | 0.7949 |
| Nodule no. vs. Nitrogenase activity | r = | 0.1831 | 0.5786 |
| Direct effect | | −0.4230 | −0.0530 |
| Indirect effect via nitrate | | 0.0034 | −0.0065 |
| Indirect effect via nodule wt. | | 0.5965 | 0.6424 |
| Indirect effect via shoot wt. | | 0.0069 | −0.0041 |
| Indirect effect via shoot protein | | | 0.0001 |
| Total | | 0.1838 | 0.5789 |
| Shoot wt. vs. Nitrogenase activity | r = | 0.4511 | 0.0966 |
| Direct effect | | 0.0120 | −0.0280 |
| Indirect effect via nitrate | | −0.0080 | 0.0022 |
| Indirect effect via nodule wt. | | 0.6900 | 0.1314 |
| Indirect effect via nodule no. | | −0.2423 | −0.0078 |
| Indirect effect via shoot protein | | | −0.0010 |
| Total | | 0.4517 | 0.0968 |
| Protein vs. Nitrogenase activity | r = | | 0.0265 |
| Direct effect | | | −0.0820 |
| Indirect effect via nitrate | | | −0.0091 |
| Indirect effect via nodule wt. | | | 0.1174 |
| Indirect effect via nodule no. | | | 0.0001 |
| Indirect effect via shoot wt. | | | −0.0004 |
| Total | | | 0.0260 |

† Correlation coefficients from analysis of each cultivar are available upon request.

ables measured. As indicated by the correlation coefficients, the most important variables affecting nitrogenase activity in the greenhouse study were nitrate, nodule fresh weight, and shoot dry weight. The negative simple correlation coefficient of nitrate with nitrogenase activity ($r_{15}$ = −0.3527) supports observations that nitrogenase activity decreases with increasing nitrate applied. However, partitioning of the effects indicated a direct effect of only −0.1220. The indirect effect via nodule fresh weight (−0.2434) accounts for most of the effects represented by the simple correlation coefficient. This indicated that the greatest impact of nitrate on $N_2$ fixation activity is through its inhibitory effect on nodule fresh weight. Effects via nodule number and shoot dry weight were very minor.

Partitioning effects of nodule fresh weight on $N_2$ fixation activity showed that the positive direct effect of nodule fresh weight (0.8730) was reduced by the negative indirect effects via nodule number (−0.2890). This indicated that a plant having many small nodules will fix less $N_2$ than a plant having fewer large nodules. One must infer the importance of nodule development and physiology to efficient fixation activity. Nitrate

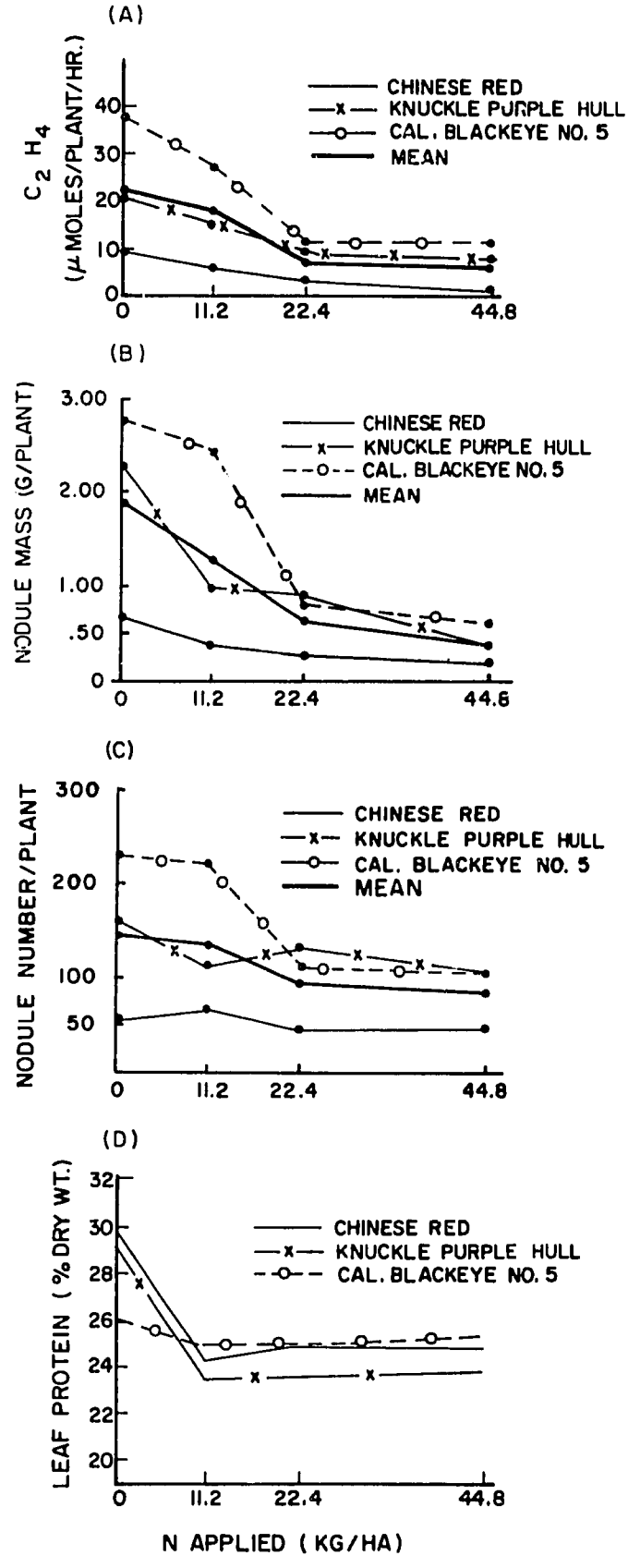


**Fig. 2. Effects of nitrate on nitrogenase activity (A); nodule fresh weight (B); nodule number (C); and leaf protein (D) in three cowpea cultivars grown in the field.**

**Table 3. Effects of nitrate on plant specific activity† in the field.**

| Cultivar | N applied (kg/ha) 0 | 11.2 | 22.4 | 44.8 | Mean |
|---|---|---|---|---|---|
| Chinese Red | 9.38 | 5.96 | 3.48 | 3.23 | 5.01 a* |
| Knuckle Purple Hull | 20.81 | 15.76 | 9.47 | 7.57 | 13.40 b |
| Cal. Blackeye No. 5 | 37.21 | 32.22 | 11.80 | 12.46 | 23.42 c |
| Mean | 22.46 | 17.98 | 8.25 | 7.09 | |

* Mean separation within columns by Duncan's New Multiple Range Test, 5% level.
† Plant specific activity = μmoles $C_2H_4$ produced per plant per hour.

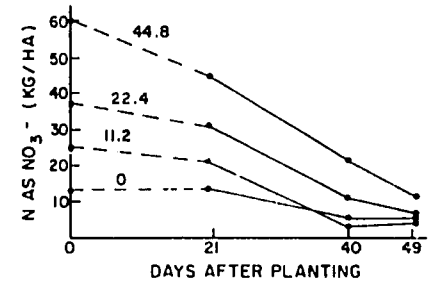


**Fig. 3. Soil nitrate levels as monitored throughout the field experiment.**

decreased nodule size but had little influence on nodule number. The low correlation (0.1831) of nodule number with nitrogenase activity is the result of a positive indirect effect via nodule fresh weight (0.5965) being greatly reduced by negative direct effects of nodule number (−0.4230). Indirect effects via nitrate and shoot dry weight were not important. The positive correlation of shoot weight with $N_2$ fixation (0.4511) was entirely accounted for by indirect effects of nodule fresh weight (0.6900) being reduced by indirect effects via nodule number (−0.2423).

*Field.* Nitrogenase activity as measured by acetylene reduction, decreased as levels of N applied increased from 0 to 22.4 kg/ha and remained low at 44.8 kg/ha (Fig. 2A). High-fixing genotypes 'California Blackeye No. 5' and 'Knuckle Purple Hull' were more severely inhibited than was 'Chinese Red.' When responses of the three cultivars were analyzed by analysis of variance, mean values for nitrogenase activity were significantly different at the 5% level using Duncan's New Multiple Range Test (Table 3). Nodule fresh weight per plant showed the same effects as nitrogenase activity. Cultivars again differed in the degree of sensitivity, with California Blackeye No. 5 retaining high nodule fresh weight at 11.2 kg/ha N but decreasing sharply at 22.4 kg/ha N applied (Fig. 2B). Nodule number per plant was not clearly affected by nitrate in cvs. Chinese Red and Knuckle Purple Hull. California Blackeye No. 5 showed a decrease in nodule number from 11.2 to 22.4 kg/ha N applied, consistent with nodule mass and nitrogenase activity (Fig. 2C). Leaf protein, as determined by micro-Kjeldahl analysis of total N, was highest for Chinese Red and Knuckle Purple Hull with no N applied and rapidly stabilized at 23 to 25% protein with supplemental N at the levels tested. Leaf protein in California Blackeye No. 5 was not affected at the levels of N tested (Fig. 2D).

Soil nitrate levels declined considerably throughout the period of the experiment (Fig. 3). Values on the day of planting were determined by adding the values for the nitrate treatments applied to those values obtained from the soil analysis. Nitrate levels declined due to leaching and utilization by plants. Soil nitrate levels were low in all treatment plots, by the time that the acetylene-ethylene assay was performed.

Correlation and path analysis was used to interpret the relationships among the variables measured. Again, linear regression equations are presented in Table 1. Path coefficients were obtained by simultaneous solution of the following equations in which '$r$' values are simple correlation coefficients and '$P$' values are standard partial regression coefficients (4):

$$r_{16} = P_{16} + r_{12}P_{26} + r_{13}P_{36} + r_{14}P_{46} + r_{15}P_{56}$$
$$r_{26} = P_{26} + r_{12}P_{16} + r_{23}P_{36} + r_{24}P_{46} + r_{25}P_{56}$$
$$r_{36} = P_{36} + r_{13}P_{16} + r_{23}P_{26} + r_{34}P_{46} + r_{35}P_{56}$$
$$r_{46} = P_{46} + r_{14}P_{16} + r_{24}P_{26} + r_{34}P_{36} + r_{45}P_{56}$$
$$r_{56} = P_{56} + r_{15}P_{16} + r_{25}P_{26} + r_{35}P_{36} + r_{45}P_{56}$$

The relationships among the variables studied are presented diagramatically in Fig. 1.

Path analysis of the field data (Table 2) showed that the most important variables affecting nitrogenase activity are nitrate, nodule weight, and nodule number. As in the greenhouse experiment, nitrogenase activity decreased with increasing nitrate. The indirect effect of nitrate via nodule weight (−0.3683) accounted for all of the effects of nitrate on nitrogenase activity (−0.3029) when the cultivars were pooled. It is interesting to note that while the general trends of each cultivar were the same, the partitioning of nitrate effects differed. Nitrate had the strongest direct effect on Chinese Red, the cultivar with the lowest $N_2$ fixation potential, but had minor impact on the high-fixing genotypes. The indirect effects of nitrate via nodule weight (−0.2753 for Chinese Red and −0.5275 for California Blackeye No. 5) suggest that this parameter may account for the observed genotypic differences in nitrate inhibition of $N_2$ fixation.

Nodule weight was correlated most highly with nitrogenase activity. Further analysis showed that all of this was due to the direct effect of nodule weight on activity. Indirect effects via nitrate, nodule number, shoot dry weight, and percent shoot protein contributed very small negative effects, reducing slightly the effect of nodule weight.

The next largest correlation coefficient was nodule number with nitrogenase activity ($r$ = 0.5786). Partitioning of this coefficient into direct and indirect effects showed that the indirect effect via nodule weight (0.6424) accounted for the entire correlation. The direct effect of nodule number and indirect effects of nitrate, shoot dry weight, and percent shoot protein all had small modifying influences, mostly counteracting the effects of nodule weight.

Genotypes differed in their correlation coefficients of nodule number with nitrogenase activity, but all were positive. Partitioning showed that the positive effect was due to indirect effects via nodule weight. In Chinese Red and California Blackeye No. 5, these

positive effects were reduced by negative direct effects of nodule number on nitrogenase activity. Knuckle Purple Hull, however, had a small positive effect of nodule number on nitrogenase activity, enhancing the correlation of nodule number with fixation activity.

Partitioning of the correlation coefficients of shoot dry weight and percent protein with nitrogenase activity showed the same general trends, although the magnitude of the effects are very much less. The indirect effects via nodule weight accounted for essentially all of the two correlation coefficients, with the direct and other indirect effects partially counteracting the positive effects of nodule weight.

## DISCUSSION

The results of these experiments differ from earlier reports that low levels of supplemental N may enhance $N_2$ fixation in cowpea (2, 3, 4, 8). The discrepancy might be due to different methods of measuring $N_2$ fixation or different forms of N. Measurement of total plant N does not distinguish between assimilation of combined N and fixed N; thus, erroneous estimation of fixed N may result. Use of the acetylene-ethylene assay might circumvent this problem.

Results of this study clearly indicated no advantage from nitrate application in promoting maximum genotypic differences in $N_2$ fixation. Inhibitory effects of soil or applied N on $N_2$ fixation in cowpea, as measured by acetylene reduction, were observed at all levels of application. This effect probably occurred through the inhibition of nodule growth rather than nodule initiation. The inhibitory effects of nitrate may occur early in plant growth and nodule development, as soil nitrate levels in the field declined rapidly during the experiment.

The impact of applied N and nodule number were important to fixation activity only through the indirect effects via nodule fresh weight. The small indirect effect of nitrate on nitrogenase activity via nodule number leads one to question the hypothesis of Tanner and Anderson (9) that soil nitrate inhibits nodule initiation and may support the view that soil N reduces nodular photosynthate import (5). The minor impact of nodule number also indicated that this parameter cannot be used to screen cowpeas for $N_2$ fixation activity. Nodule weight was the main parameter contributing to $N_2$ fixation activity in both greenhouse and field, while nodule number was important mainly through correlation with nodule weight.

## ACKNOWLEDGMENT

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