

Correlations and Path Analysis in Segregating Cowpea Generations Regarding Biological Nitrogen Fixation

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Abstract

The objective of this research was to evaluate the correlations between variables related to the biological nitrogen fixation (BNF) in segregating generations of cowpea and to unfold these correlations in direct and indirect effects, through path analysis. An outdoor bench experiment was conducted at Carpina Experimental Sugarcane Station of, located at the Zona da Mata region of the State of the Pernambuco, Northeast of Brazil (Federal Rural University of Pernambuco), between March and April 2016. The seeds were planted in 20 cm × 30 cm polyethylene bags, using a substrate composed of a mixture of vermiculite and sand washed in a ratio of 1:1. *Bradyrhizobium* references, recommended for culture, were used as a mixture of two strains. Parental and F2, F3 and F4 generations were evaluated in a randomized block design with four replicates. Data collection was performed 45 days after the emergency (DAE). Phenotypic correlations and path analysis of the number of nodules per plant (NN), nodules dry mass (NDM), dry roots mass (DRM), dry mass of aerial part (DMAP), nodulation efficiency (NODE) and nitrogen accumulated in the aerial part (NAAP). The phenotypic correlations between the variables related to the BNF showed high magnitudes, demonstrating that there is a great influence of each of the variables on the others, furthermore the path analysis of the coefficients indicated that all the primary components (NN, NDM, DRM, DMAP and NODE) must be considered when it is desired to increase the NAAP in segregating generations of cowpea.

Keywords: *Bradyrhizobium*, strains, progenies, *Vigna unguiculata*

1. Introduction

Cowpea (*Vigna unguiculata* L. Walp.) is a legume consumed as green and dry grains that has a high protein content, short cycle for maturity and drought tolerance. It is a dicotyledonous species of African origin, known in several regions of Brazil as feijão-de-corda, feijão-macassar, feijão-de-praia e feijão-miúdo. Cultivated in tropical and subtropical regions of Africa, Asia, America, Europe and Oceania (Freitas et al., 2016; Freire Filho et al., 2011), this crop has great socioeconomic importance, being the main source of vegetable protein for rural populations.

With a wide adaptation to the climate and soil, its cultivation is carried out in about 45 countries (Abate et al. 2012), with Africa responsible for more than 95% of world production (FAO, 2017). Brazil is the world's third largest producer of cowpea, after Nigeria and Niger. These three countries together represent 82.46% of the harvested area and 81.92% of world production (FAO, 2017; CONAB, 2017).

In Brazil, the main producing regions are North and Northeast with expansion to the Midwest and Southeast regions (Rocha et al., 2009). In the 2016/2017 harvest, cowpea was the second most cultivated crop in this country, with an area of 1,409.3 hectares, estimated production of 713.0 thousand tons and average productivity of 506 Kg ha⁻¹ (CONAB, 2017).

The cowpea bean is capable of nodulating with several genera of bacteria of the rhizobia group (Zilli et al., 2009). The use of strains is low cost and can promote increased productivity through effective symbiosis (Gualter et al., 2008; Costa et al., 2011).

The study of correlations is very important in breeding programs, especially when the selection of a desirable trait presents difficulties due to low heritability and/or measurement or identification problems (Nogueira et al., 2012). Correlation estimates and path analysis between components of biological nitrogen fixation (BNF) allow breeders to determine the most efficient procedure in conducting an improvement program for biological nitrogen fixation.

Correlation represents the joint variation between two characteristics and measures the intensity of association and linear direction or not between them. However, only the path analysis allows understanding the relationships between the variables that define or influence given characteristics, unfolding correlations into direct and indirect effects, measuring the influence of variables on the main characteristic (Wright, 1921).

For the correlation between variables to provide biologically correct estimates and interpretations, it is necessary to perform multicollinearity analysis between the independent variables. In addition, the researcher must establish in advance the importance of the set of variables being studied (Cruz, Regazzi, & Carneiro, 2012).

The BNF is essential for the development of cowpea (Silva et al., 2011), however, studies evaluating the correlations between BNF variables in segregating generations of this crop were not found in the literature. In this sense, the objectives of this study were to evaluate the phenotypic correlations between variables related to BNF in cowpea cultivars, used as genitors, and in their segregating generations and to perform the analysis of path analysis with nitrogen accumulation in the aerial part of the plants as the main character.

2. Material and Methods

The experiment was conducted on outdoor platform in an area of the Carpina Sugarcane Experimental Station-EECAC (Federal Rural University of Pernambuco-UFRPE), Carpina, PE (7°51'04"S; 35°14'27"W; 178 m asl.). The climate was classified as Tropical Rainy with dry summers in the Köppen classification and the total rainfall during the experimental period was 426.1 mm, favoring the vegetative development of the crop.

The seeds of the parents and segregating generations were provided by the Cowpea Genetic Improvement Program, from Agronomic Institute of Pernambuco-IPA. As female genitor the cultivar BRS Pajeú (P1) was used and as male genitor the cultivar Patativa (P2), both with productive and commercial standard characteristics. The F2, F3 and F4 generations were obtained by performing the P1 × P2 cross and the self fecundations of the F1, F2 and F3 generations.

The P1 × P2 crossing was performed in 2010, at the IPA headquarters in Recife, PE. Each year, successive self fecundations from F1 to F5 were performed. Segregating generations were conducted by the single pod descent method from the F2 to F5 generation, when the lineages were opened. All seeds from the F1 generation were used to produce the F2 generation. From the F2 generation, the seeds from the harvested pods formed a bulk with the seeds from all plants of the same cross. From each bulk a sample was taken to be sown, composing the plants of the next generation. The remaining seeds from each cross were stored for future trials.

For implementation of this experiment, sowing was performed on April 7, 2016 and plant collection was performed on May 26, 2016. At planting, two seeds were placed per bag, for better seedling development and to take advantage of the most developed seed at the time of thinning, which occurred at 15 days after the emergency (DAE), leaving one plant per bag.

The experimental design adopted was a randomized block design with four repetitions, and five treatments were evaluated (the two parents and the F2, F3, and F4 generations). The plots consisted of 24 plants from each of the genitor and 76 plants from each of the F2, F3 and F4 generations. The seeds were planted in 20 cm × 30 cm polyethylene bags, using as substrate a mixture composed of vermiculite and washed sand in a 1:1 ratio, each bag being superimposed on a Styrofoam plate. Irrigation was carried out during planting, leaving the substrates saturated until the Styrofoam dishes were filled, which received 50 mL of Hoagland's nutritive solution without nitrogen every two days (Hoagland & Arnon, 1950).

The *Bradyrhizobium* strains BR 3267 and BR 3262 used in the experiment were provided by Embrapa Agrobiologia, Seropédica, RJ. For inoculum preparation, both strains were repotted into erlenmeyers containing liquid YM medium (Mannitol and yeast extract) and incubated under agitation (220 rpm) at 28 °C for 96 hours and then stored in a refrigerator. The inoculation was carried out after the cotyledons had fallen from most plants and consisted of adding 2.5 mL of the inoculum containing the mix of strains BR 3267 and BR 3262 in each of the bags.

Nodulation data were collected on individual whole plants. These plants were separated into root and aerial part by a cut at the cotyledon insertion point at 45 DAE. The roots were washed over a sieve to avoid loss of nodules, and these were detached from the roots for determination of the number of nodules per plant (NN), nodule dry mass (NDM) and dry root mass (DRM). The nodules, roots, and the fresh aerial part of the plants were packed in paper bags and placed in an air circulation oven at 60-70 °C until reaching constant weight (+/- 72 hours). After drying, the aerial part was weighed to determine the dry mass of the aerial part (DMAP). Afterwards it was ground in a Willey type knife mill and submitted to wet digestion for quantification of total N by the Kjeldahl method (Silva, 2009).

The nitrogen accumulated in the aerial part (NAAP) was calculated from the total N content multiplied by DMAP and nodulation efficiency (NODE) was determined by dividing the total N of the aerial part by NDM (Melo & Zilli, 2009). Nitrogen derived from biological fixation (NDBF) is equal to NAAP, because inoculation of diazotrophic bacteria was the only source of nitrogen for plant nutrition.

For the determination of phenotypic correlations and path analysis, the variables evaluated were: NN, NDM, DRM, DMAP, NAAP and NODE. For the path analysis, the NAAP variable was considered as the main variable and the others as explanatory or secondary variables. The estimates of phenotypic correlation between BNF variables and the coefficient of determination (R²) were determined by the mathematical model of Cruz, Regazzi, and Carneiro (2012).

The significance of phenotypic correlations was estimated by the t-test with n-2 degrees of freedom, where n corresponds to the number of generations evaluated. The unfolding of these correlations into direct and indirect effects was performed by means of the trail analysis developed by Wright (1921).

We performed the diagnosis of multicollinearity of the X'X matrix in the explanatory variables, based on the condition number (CN, the ratio between the largest and smallest eigenvectors of the matrix) and the determinant value of the correlation matrix between the variables, established by Montgomery and Peck (1992), where, NC < 100 is not a serious problem (weak multicollinearity); 100 < NC < 1000 shows moderate to strong multicollinearity and NC > 1000, severe multicollinearity occurs.

To circumvent the effects of multicollinearity, without the need to discard variables, the ridge regression method was used (Cruz, Regazzi, & Carneiro, 2012) where a variable was added (k = 5,2568) to the diagonal of the X'X matrix. With this procedure, it was possible to obtain an NC less than 100, which characterizes weak multicollinearity and does not compromise the trail analysis. Subsequently, the correlations were performed. All analyses were performed using the computer program Genes (Cruz, 2013).

3. Results and Discussion

The estimates of phenotypic correlations between pairs of variables were predominantly of high magnitude, (above 0.7) (Table 1) and positive for most variables, suggesting that an increase in any of these components, with the exception of NODE.

Table 1. Estimates of phenotypic correlation coefficients (r_f) between pairs of variables number of nodules per plant (NN), nodule dry mass (NDM), dry root mass (DRM), dry mass of the aerial part (DMAP), nitrogen accumulated in the aerial part (NAAP) and nodulation efficiency (NODE) in five generations of cowpea

Variables	NDM	DRM	DMAP	NAAP	NODE
NN	0.955**	0.929*	0.882*	0.939*	-0.705 ^{ns}
NDM		0.975**	0.792 ^{ns}	0.856 ^{ns}	-0.800 ^{ns}
DRM			0.840 ^{ns}	0.887*	-0.689 ^{ns}
DMAP				0.876*	-0.532 ^{ns}
NAAP					-0.428 ^{ns}

Note. ** and *: significant at the level of 1 and 5% probability, respectively by the t test; ^{ns}: not significant.

The estimates of the phenotypic correlation coefficients for the pairs of characteristics at levels of significance and magnitude are considered low when they present values below 0.4; averages between 0.4 and 0.7; and high values greater than 0.7 (Santos et al., 2015). Phenotypic correlations were used because the phenotype is considered to be the basis for selection. The phenotypically correlated variables have practical selection value because they have a high genetic component in their phenotypic expressions, providing gains through visual selection (Andrade et al., 2010).

The primary variables (NN, DRM and DMAP) showed high positive and significant correlations with the basic variable (NAAP). In interpreting correlations, three aspects must be considered: magnitude, direction and significance. Positive correlation coefficient estimates indicate a tendency for one variable to increase when the other also increases, while negative correlations indicate a tendency for one variable to increase while the other decreases (Nogueira et al., 2012). The results found that the primary components are directly related to the basic variable NAAP, however they show negative correlation with the variable NODE.

NN was the variable with the greatest direct influence on NAAP, as it showed a significant and positive correlation of the highest value (0.939). For this character, positive and significant ($P < 0.01$ or $P < 0.05$) correlations were observed with most variables (NDM, DRM and DMAP), except NODE, suggesting that selecting individuals with high NN values may lead to gains in NDM, DRM, DMAP and NAAP.

As for the significance of the data, our results were contrary to those obtained by Frigo et al. (2014), who analyzed the inoculation of rhizobia strains in cowpea 'BRS New Era' (including the commercial strain BR3267) and obtained a positive but non-significant correlation of the NN variable with the NDM, DRM, DMAP and NAAP variables.

A negative and non-significant correlation was observed between the NN and NODE variables, indicating that for this correlation, indirect selection is not efficient, that is, selection based on NN would not result in significant changes to the NODE variable.

The NDM character showed positive and significant correlation with MRD ($P < 0.01$), positive but not significant with DMAP and NAAP, and negative and not significant with NODE. Studying the agronomic efficiency of rhizobia strains inoculated into cowpea bean in the Cerrado, Chagas Júnior et al. (2010) observed an increase in DMAP as there was an increase of nodules dry mass (NDM), While Campanharo et al. (2010) reported a significant positive correlation between the variables NDM and NAAP.

DRM showed positive and significant correlation with NAAP, positive but not significant correlation with DMAP and negative correlation with NODE. NAAP showed positive and significant correlations with NN, DRM and DMAP, positive but not significant correlation with NDM and negative with NODE. For the NAAP character, Frigo et al. (2014) reported divergent results to ours, with a negative and non-significant correlation with DMAP and DRM, coinciding only the correlation of NDM with NAAP that was also positive, but not significant.

We observed that the variable NODE was the only one that correlated negatively with the others. With regard to cowpea improvement in relation to BNF, this is a relevant finding, because in this case it indicates that selection based on any of these variables would not result in significant increases in NODE.

By estimating the correlations in direct and indirect effects of the explanatory variables on the primary character NAAP (Table 2), we verified that the basic character was positively influenced by all these variables. This highlights the importance of analyzing these variables in breeding programs that seek to explore the efficiency of biological nitrogen fixation in cowpea. The knowledge of the path analysis allows establishing the most efficient selection strategy to increase the genetic improvement of a crop.

Table 2. Estimates of direct and indirect effects of explanatory components of biological nitrogen fixation on the character nitrogen accumulated in the aerial part (NAAP), in five generations of cowpea.

Variables	Estimates	Variables	Estimates
NN	0.6313	NDM	0.2762
Direct effect on NAAP	0.2639	Direct effect on NAAP	0.6031
Indirect effect via NDM	-0.1554	Indirect effect via NN	-0.1631
Indirect effect via DRM	0.1562	Indirect effect via DRM	0.1404
Indirect effect via DMAP	-0.3004	Indirect effect via DMAP	0.3409
Indirect effect via NODE	0.9396	Indirect effect via NODE	0.8565
Total	0.6313	Total	0.2762
DRM		DMAP	0.1772
Direct effect on NAAP	0.1673	Direct effect on NAAP	0.5566
Indirect effect via NN	0.5863	Indirect effect via NN	0.2188
Indirect effect via NDM	0.2692	Indirect effect via NDM	-0.1407
Indirect effect via DMAP	0.1490	Indirect effect via DRM	-0.2266
Indirect effect via NODE	-0.2938	Indirect effect via NODE	0.8762
Total	0.8870	Total	0.1772
NODE	0.4259		
Direct effect on NAAP	-0.4453	Coefficient of determination (R^2)	0.9513
Indirect effect via NN	-0.2210	k-value used in the analysis	5.2568
Indirect effect via NDM	0.1154	Effect of residual variable	0.0093
Indirect effect via DRM	-0.0942		
Indirect effect via DMAP	-0.4276		
Total	0.4259		

The NN character contributed most directly to explain nitrogen accumulated in the aerial part (NAAP), as it showed a total effect of medium magnitude ($0.4 < r < 0.7$) with a direct effect of 0.6313, offsetting the negative indirect effects that occurred via MRD (-0.1554) and NODE (-0.3004), through the positive indirect effects via NDM (0.2639) and DMAP (0.1562), resulting in a positive correlation coefficient estimate (0.9396). This demonstrates that NN is an efficient character for indirect selection in order to obtain significant gains in BNF in parents and segregating generations of cowpea. Thus, the breeder interested in increasing NAAP in cowpea should increase selection pressure on NN, but cannot disregard the other variables involved in BNF.

The variables NDM, DRM and DMAP showed positive relationships with the basic character NAAP, but their direct effects were of low magnitudes. Thus, it can be seen that these correlations occurred due to the great influence of NN, because the indirect effects on NAAP via this variable were high.

The NDM variable showed a positive direct effect of low magnitude (0.2762), but due to the positive effects via the explanatory variables NN (0.6031), DMAP (0.1404) and NODE (0.3409) the negative effect via DRM (0.1631) was offset, resulting in a correlation of high magnitude (0.8565).

For the DRM character the direct effect was positive and of low magnitude (0.1673), but due to the positive effects via the explanatory variables NN (0.5863), NDM (0.2692) and DMAP (0.1490) the negative effect via NODE (0.2938) was offset, resulting in a high value correlation with NAAP (0.8870).

For the explanatory variable DMAP the direct effect was also of low magnitude (0.1772), but the positive indirect effects caused by the variables NN (0.5566) and NDM (0.2188), offset the negative indirect effects of DRM (-0.1401) and NODE (-0.2266), resulting in a positive correlation estimate (0.8762).

The positive direct effect of NODE (0.4259), despite presenting medium magnitude and relying on the positive indirect effect via MRD (0.1154), did not offset the negative indirect effects caused via NN (-0.4453), NDM (-0.2210) and DMAP (-0.0942), which resulted in a negative estimate of the value of the correlation coefficient on NAAP (-0.4276).

Coefficients of determination with high values indicate that the variation in the main character was largely determined by the explanatory variables (Freitas et al., 2016). In this work, the coefficient of determination of the trail analysis was higher than that reported by Moura et al. (2012), Almeida et al. (2014), Santos et al. (2014),

and Ribeiro et al. (2016), in similar studies. The coefficient of determination of the main variable NAAP, originated by the primary components, showed that more than 95% of this character was determined by the contributions of the explanatory variables and only 0.93% by the residual variable. Thus, the adopted explanatory model expressed the cause-and-effect relationship between the primary variables and nitrogen accumulation in the aerial part.

4. Conclusions

The phenotypic correlations between the BNF-related variables show that there is a strong influence of each variable on the others.

The path analysis proved to be adequate to explain the relationship between the primary components and the nitrogen accumulation in the aerial part, considering the high estimate of the coefficient of determination (R^2) and the low residual effect.

The direct and indirect effects of the primary variables indicate that it is possible to select plants with higher NAAP content when they have higher values of the explanatory variables.

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