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Effects of *Phaseolus vulgaris* QTL in controlling host-bacteria interactions under two levels of nitrogen fertilization*

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Abstract

Molecular markers were used to estimate the effect of mineral nitrogen on the phenotypic expression of quantitative trait loci (QTL) controlling the number of *Rhizobium* nodules (NN) and resistance to *Xanthomonas axonopodis* pv. *phaseoli* in the common bean. Recombinant inbred lines derived from a BAT-93 x Jalo EEP558 cross were grown in a greenhouse in the absence or presence (5 mM NH_4NO_3) of nitrogen. Resistance to *Xanthomonas* was assessed as diseased leaf area (DLA) and the number of nodules was obtained by direct counting. Analyses of variance were used to detect significant associations between 85 marker loci from 12 linkage groups (LG) and quantitative traits. In the absence of nitrogen, 15 and 11 markers, distributed over 7 and 5 LG, showed a significant association with NN and DLA, respectively. The combined percentage of phenotypic variance explained by the marker-loci and QTL associations was 34% for NN and 42% for DLA. In the presence of nitrogen, there were only five significant associations for NN and eight for DLA, which explained 28 and 26% of the total phenotypic variance, respectively. The effects of some QTL were detected only at a certain level of nitrogen. The contribution of parental alleles at two NN QTL was dependent on the level of nitrogen. Four QTL were associated with both the number of *Rhizobium* nodules and resistance to *Xanthomonas*, suggesting a common genetic control of responses to bacterial infections in the common bean. Despite the dramatic environmental interactions noted with some QTL, in other cases the phenotypic effects were not affected by the amount of nitrogen. The stability of the latter QTL may be relevant when breeding cultivars adapted to variable soil fertility.

INTRODUCTION

Increased levels of biological nitrogen fixation and resistance to common bacterial blight (CBB), caused by *Xanthomonas axonopodis* pv. *phaseoli* (Smith) Vauterin, Hoste, Kerstens & Swigs, are two of the most important agricultural traits of the common bean (*Phaseolus vulgaris* L.) in the tropics. The development of cultivars with increased nodulation following infection with *Rhizobium* could reduce the amount of mineral nitrogen normally used in commercial fields to increase yields (Tsai *et al*., 1993). Moreover, higher levels of resistance to CBB, a major threat in tropical areas with a warm, humid climate, could reduce losses in yield and seed quality commonly associated with this disease (Bianchini *et al*., 1997).

The ability of *P. vulgaris* to establish a successful symbiotic relationship with *Rhizobium tropici* is highly dependent on the level of mineral nitrogen supplied, with the usual recommended levels generally suppressing nodule formation (Graham, 1981). Thus, it is not possible to profit from mineral and atmospheric nitrogen sources at once, unless cultivars with nitrogen-tolerant, nodulation-related genes are developed. Resistance to CBB is also subject to environmental interactions. Mohamed and Coyne (1995) reported that photoperiod influences the expression of CBB, with susceptible tropical genotypes showing a higher level of resistance when grown under sub-tropical conditions. So far, the effect of nitrogen on the phenotypic expression of resistance genes of *Phaseolus* to *Xanthomonas* has not been studied. This is an important area of research, since in tropical areas common beans are cultivated under a variety of geographic and technological conditions which affect the amount of nitrogen available to both host and pathogen.

The traditional genetic analysis and breeding for an increased number of *Rhizobium* nodules (NN) and resistance to *X. axonopodis* pv. *phaseoli* is hampered by the fact that both traits are under oligogenic control. In such cases, molecular markers have been used successfully to identify individual oligogenes and to study their phenotypic effects (see Young, 1996 for references). Nodari *et al*. (1993b), for example, found at least four quantitative trait loci (QTL) in *P. vulgaris* which accounted for 50 and 75% of the phenotypic variation in NN and CBB resistance, respectively. One of these QTL was found to be associated with both traits, and was also reported to control resistance to the variant fuscans of *X. axonopodis* pv. *phaseoli* (Boscariol *et al*., 1998). Despite these important findings, much remains to be learned of the phenotypic stability of

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these QTL in other genetic backgrounds and under different environmental conditions, especially those related to soil fertility. The objective of the present study was to determine the effect of mineral nitrogen on the phenotypic expression of QTL associated with NN and resistance to CBB.

MATERIAL AND METHODS

Plant materials

$F_{8:9}$ recombinant inbred lines (RILs) were generated by single-seed descent from a BAT-93 x Jalo EEP558 F_2 population, with each line descending from a different F_2 plant (Freyre *et al*., 1996). BAT-93 is a Middle American breeding line which displays lower numbers of nodules after inoculation with *Rhizobium tropici* and is more resistant to *X. axonopodis* pv. *phaseoli* than the Andean landrace Jalo EEP558 (Nodari *et al*., 1993a). These RILs have been used in multiple studies and are now considered a core mapping population of the common bean (Gepts, 1998).

Greenhouse trials

Two trials (A and B) were conducted simultaneously in a greenhouse. The trials were identical, except that in one trial plants were irrigated with a nitrogen-free nutritive solution, whereas in the second they were irrigated with the same nutritive solution supplemented with mineral nitrogen (5 mM NH_4NO_3). In both trials, the same 51 RILs were screened for CBB resistance and NN using a randomized complete block design consisting of four blocks. Plots consisted of one plant per RIL. Prior to sowing, the seeds were disinfected by immersion in 70% ethanol for 3 min and in 0.5% NaClO for 3 min after which they were then thoroughly rinsed with distilled water. Two seeds were sown per 1.5 l Leonard jar previously soaked in 70% ethanol. The outside surface of the jar was wrapped in aluminum foil. The substrate consisted of an autoclaved mixture of sand and vermiculite (1:1, v/v), pH = 6.9, and was irrigated as needed with 100 ml of 1/3-strength nitrogen-free Sarruge's solution (Sarruge, 1975) in trial A and the same amount of this solution containing 5 mM NH_4NO_3 in trial B. Seedlings were inoculated with 20 ml of a (10^8 c.f.u./ml) liquid culture of *R. tropici*, strain UMR-1899, four days after germination. After inoculation, the surface of the substrate was covered with a thin layer of autoclaved sand. Eighteen days after germination, two leaflets of the first trifoliate leaf were inoculated with a (10^8 c.f.u./ml) liquid culture of *X. a*. pv. *phaseoli* strain W-18. The inoculum preparation and inoculation of both bacteria were done as described by Nodari *et al.* (1993b). The symptoms of CBB were evaluated 13 days after inoculation with the pathogen. Lesioned area (cm^2) of each leaflet was measured according to Camargo *et al*. (1995) and averaged to represent the experimental unit in statistical analyses. Thirty-two days after germination, the roots were harvested, carefully wrapped in tissue paper, placed in plastic bags, and stored at 10°C until the number of nodules was assessed. Controls in both trials consisted of parental lines inoculated only with *Rhizobium* and with *Rhizobium* and *Xanthomonas*. The mean diseased leaf area (DLA) and NN of the four replicates were used for QTL mapping. The data were tested for normality using the Shapiro-Wilk correlation test prior to QTL analysis. Trait means of the parents and RILs were compared by the F-test and Duncan's multiple range test using the computer program STATISTICA (StatSoft, USA). Standardized residual plots were inspected for the absence of variance homogeneity and the need for data transformation.

Linkage analysis

A linkage map with 85 RAPD, RFLP and SCAR marker loci was used in this study. Marker-genotypes of the RILs were obtained from Freyre *et al*. (1996), and construction of the linkage map is described elsewhere (Boscariol *et al*., 1998). This map was assembled based on a subset of marker scores from 91 RILs, including those used in this study, chosen to be representative of the consensual map described by Freyre *et al*. (1996).

One-way analyses of variance were conducted for all pairwise combinations between marker loci and quantitative traits using the general linear model (Edwards *et al*., 1987). Linkage between a marker locus and a quantitative trait was assumed when there was a significant difference ($P \leq 0.01$) between the mean phenotypic value of the trait of the two marker genotypes. The proportion of the phenotypic variance explained by the marker-trait association was estimated by the coefficient of determination (R^2; Edwards *et al*., 1987). When multiple significant linked loci were found, the significance of their additive effects was evaluated in multiple regression models to establish the presence of linked QTL (Austin and Lee, 1996). A multiple regression model was also used to estimate the total phenotypic variation of a trait explained by the additive effects of all detected QTL. This model included a single marker with the highest R^2 value from each QTL. All statistical analyses were performed with the aid of the computer program STATISTICA (Statsoft, USA).

RESULTS

QTL mapping in trial A (no nitrogen supplied)

The distribution of the mean DLA and NN of RILs was non-normal and residual plots of the analyses of variance indicated increased variance for higher values (data not shown). Thus, SQRT (NN) and LOG (DLA) transformations were used to randomize the distribution of standardized residuals, and all marker-trait analyses were done using transformed data.

Analysis of transformed NN and DLA values indicated significant variation among entries (RILs and parental con-

trols), with some RILs having mean NN and DLA significantly lower than those of the parental controls, indicating the presence of transgressive segregation (Table I). No transgression of the higher parental values was observed for either trait. There was a significant difference in mean NN between BAT93 and Jalo EEP558 both in the *Xanthomonas* inoculated and non-inoculated control treatments. However, there was no difference in the mean NN of the two parental controls (Table I).

Table I - Diseased leaf area (DLA) and nodule number (NN) in the absence (trial A) and presence (trial B) of nitrogen (5 mM NH_4NO_3) in parental line controls (BAT and Jalo) and recombinant inbred lines (RILs).

	Trial A		Trial B	
Genotypes	DLA (cm^2)	NN	DLA (cm^2)	NN
Jalo[1]	10.8[a3]	267.9[a]	9.5[a]	270.1[a]
BAT[1]	4.2[b]	167.6[b]	4.2[b]	127.8[b]
Jalo[2]	-	347.7[a]	-	244.9[a]
BAT[2]	-	182.5[b]	-	149.7[b]
RIL (range)	1.5-12.5	95.6-392.1	1.5-13.6	55.3-270.1

[1]Parental controls inoculated with *Rhizobium* and *Xanthomonas*. [2]Parental controls inoculated only with *Rhizobium*. [3]Unless otherwise indicated all values are means. Values with the same letters within a column did not differ significantly (P = 5%; Tukey test).

Analyses of variance revealed significant associations between the mean NN and 15 markers from seven linkage groups (LG) (Table II). The percentage of phenotypic variation explained by these associations ranged from 4 to 11%. Multiple regression analyses suggested the presence of one QTL per LG. The additive effects of all putative QTL explained 34% of the total phenotypic variation in NN when combined in a multi-locus model. Alleles from Jalo EEP558 contributed to higher NN values in most cases, except for markers on LG 5 and 10 (Table II).

Significant associations were found between DLA and 11 marker-loci from 5 LG (Table III). The percentage of phenotypic variation explained by these associations ranged from 5 to 14%. Multiple regression analyses suggested the presence of a single QTL per linkage group. The total percentage of phenotypic variation explained by the additive effects of all QTL was 42%. For markers on LGs 7, 9, 10 and 11, alleles from the resistant parent BAT93 contributed to resistance, whereas for markers on LG 3 the opposite was true (Table III).

QTL mapping in trial B (5 mM NH_4NO_3 supplied)

The distributions of the mean DLA and NN of RILs grown in the presence of nitrogen were also non-normal. Higher frequencies of intermediate sized lesions were ob-

Table II - Markers significantly associated ($P \leq 0.01$) with the number of *Rhizobium* nodules (NN) in *Phaseolus vulgaris* inoculated with both *Rhizobium tropici* and *Xanthomonas axonopodis* pv. *phaseoli* and grown with 0 mM or 5 mM NH_4NO_3 (trials A and B, respectively), based on RILs of a BAT x Jalo cross.

		Trial A				Trial B			
Linkage group	Marker	R^2	BB[a]	JJ[b]	P	R^2	BB	JJ	P
LG 2	D1367	0.04	182	219	< 0.001	-	-	-	-
	D1287	0.08	179	228	< 0.001	-	-	-	-
LG3	RoF10a	0.08	178	227	<0.001	-	-	-	-
	D1132	-	-	-	-	0.06	159	123	<0.001
LG 5	Aco-2	0.08	207	164	< 0.001	-	-	-	-
	D1157	0.05	218	177	< 0.001	-	-	-	-
	D1251	-	-	-	-	0.05	122	153	< 0.001
LG 6	D1086	0.08	177	226	< 0.001	-	-	-	-
LG7	RoF1b	0.04	179	216	<0.010	-	-	-	-
	Chi	0.11	177	233	<0.001	0.05	119	151	0.006
LG 10	D1580	0.06	220	179	< 0.001	0.05	153	122	0.004
LG 11	D1308	-	-	-	-	0.05	146	114	< 0.001
	D1291	0.04	184	219	< 0.001	-	-	-	-
	RoJ17c	0.05	180	217	<0.001	-	-	-	-
	P2027	0.05	199	225	<0.010				-
	RoJ9a	0.05	178	217	<0.001	-	-	-	-
	D1630	0.04	185	218	0.005	-	-		-
	RoN9b	0.04	180	216	0.003	-	-		-

[a]Mean NN of RIL homozygous for BAT alelles. [b]Mean NN of RIL homozygous for Jalo alelles.

Table III - Markers significantly associated ($P \leq 0.01$) with diseased leaf area (DLA) in *Phaseolus vulgaris* inoculated with both *Rhizobium tropici* and *Xanthomonas axonopodis* pv. *phaseoli* and grown with 0 mM or 5 mM NH_4NO_3 (trials A and B, respectively), based on RILs of a BAT x Jalo cross.

		Trial A				Trial B			
Linkage group	Marker	R^2	BB[a]	JJ[b]	P	R^2	BB	JJ	P
LG2	D1287	-	-	-	-	0.08	5.76	3.84	< 0.001
LG3	RoF7c	0.05	5.25	3.97	< 0.001	-	-	-	-
	RoAD19a	0.14	5.85	3.61	< 0.001	-	-	-	-
	RoF10a	0.06	5.54	4.02	0.005	-	-	-	-
LG7	Bng204	0.09	4.17	5.91	< 0.001	-	-	-	-
	D1861	0.12	3.47	5.24	< 0.001	-	-	-	-
	RoF1b	0.12	3.55	5.42	< 0.001	-	-	-	-
	Chi	0.08	3.81	5.52	< 0.001	-	-	-	-
LG9	D1096	0.05	3.8	4.97	0.001	-	-	-	-
LG10	RoE9a	0.06	3.79	5.14	0.002	0.08	4.24	6.45	< 0.001
	RoD4b	0.09	3.69	5.41	0.001	0.09	4.14	6.22	< 0.001
LG11	D1308	0.06	4.94	6.63	< 0.001	-	-	-	-
	D1291	-	-	-	-	0.11	4.21	6.54	< 0.001
	RoJ17c	-	-	-	-	0.10	4.34	6.63	< 0.001
	P2027	-	-	-	-	0.10	4.23	6.46	< 0.001
	RoJ9a	-	-	-	-	0.08	4.39	6.35	0.003
	D1630	-	-	-	-	0.09	4.42	6.44	0.001

[a]Mean DLA of RIL homozygous for BAT alelles. [b]Mean DLA of RIL homozygous for Jalo alelles.

served compared to the no-nitrogen trial, while the distribution of mean NN was severely skewed towards lower values (data not shown). The data were transformed as in trial A. Analysis of transformed DLA and NN data indicated significant variation among entries (RILs and parental controls). As in trial A, significant differences were found between the mean DLA and NN of BAT93 and Jalo EEP558 in *Xanthomonas* inoculated and non-inoculated controls, but no difference was observed within either control (Table I). Significant transgression of both parental means was found for DLA, while for NN, transgression was observed only towards the lower value.

Analyses of variance indicated five significant marker-NN associations, with R^2 values ranging from 5 to 6%. Two of these markers (*Chi* and *D1580*) were also significant in trial A, whereas the other three, although only significant in trial B, were linked to the significant ones from trial A (Figure 1; Table II). The additive effects of all QTL explained 28% of the total phenotypic variation in NN. For QTLs on LGs 5 and 7, alleles from BAT93 reduced the number of nodules, whereas on the remaining LG BAT93 alleles acted in the opposite way.

Three QTL influencing DLA were detected when plants were grown in the presence of nitrogen. Their R^2 values ranged from 8 to 11%, and their combined effect explained 26% of the total phenotypic variance of this trait (Table III). The effect of QTL from LG 2 was significant in this trial only and, in contrast to the other two QTL, BAT93 alleles contributed towards susceptibility.

DISCUSSION

One of the advantages of using molecular markers to study the inheritance of quantitative traits is the possibility of studying genotype vs. environment interactions on a gene-to-gene basis. In maize, for example, Bubeck *et al*. (1993) found that the effect of resistance genes on gray leaf spot was generally inconsistent when evaluated in different environments, whereas Camargo *et al*. (1995) reported that the effects of cabbage QTL in controlling adult-plant resistance to black rot could also be detected in a greenhouse trial using young plants, indicating the consistency of these loci in different environments, inoculation methods and plant ages.

As shown above, the nitrogen supply had a marked effect on the phenotypic expression of QTL controlling both NN and CBB resistance. The total explained variance of both traits was lower when plants were grown in the presence of nitrogen because of the lower R^2 values of individual markers. In addition, the number of significant marker-trait associations was decreased. Thus, for instance, the number of markers associated with NN was 15 and 5, and with CBB resistance, 11 and 8, for trials A and B, respectively.

The expression of some QTL was nitrogen-independent, while others were detected only with a certain nitrogen supply. All the NN QTL detected in trial B were in the same LG as those detected in trial A, suggesting that they are indeed the same, although one cannot rule out the possibility of linkage. On the other hand, two NN QTL detected

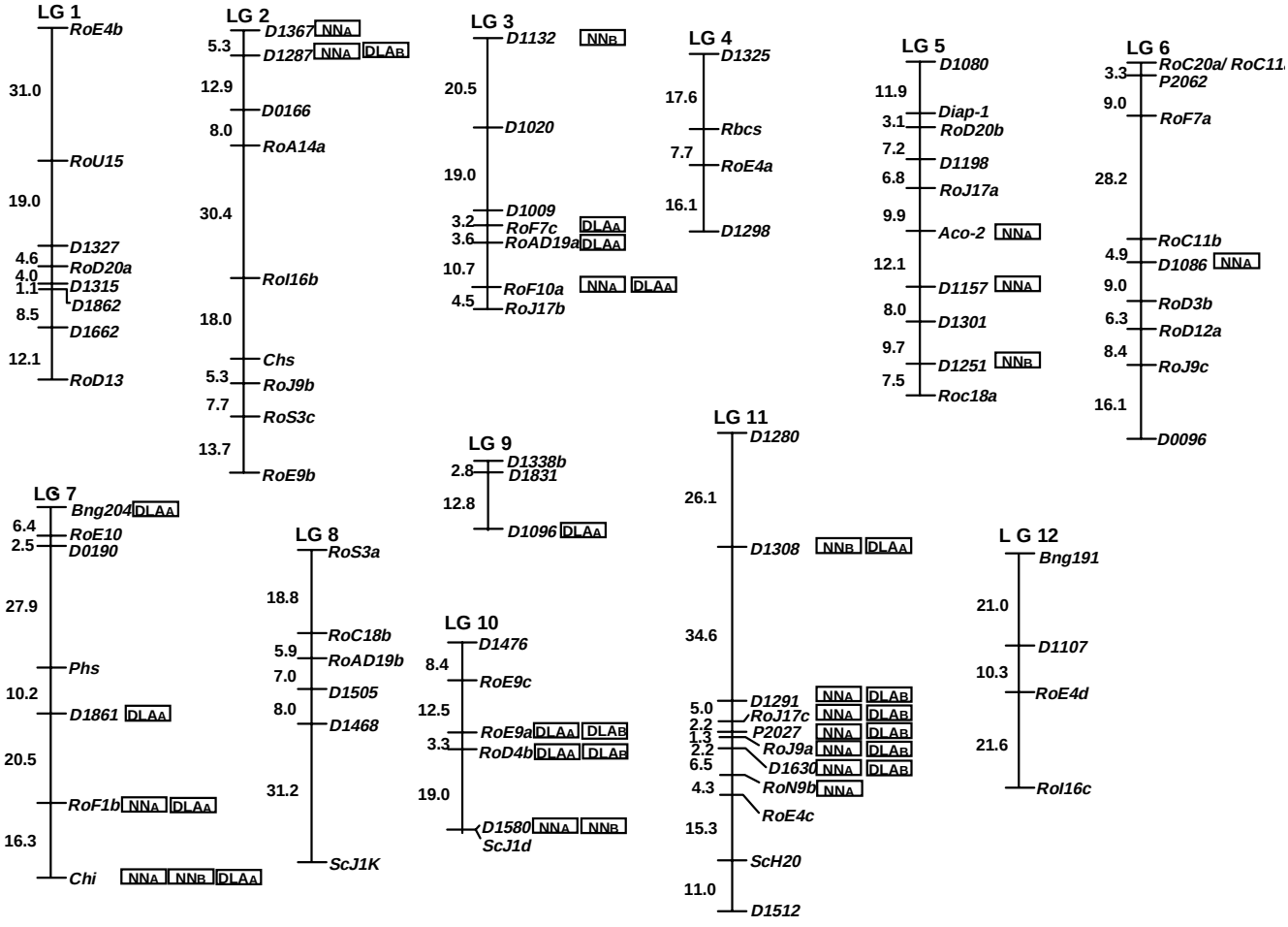

Figure 1 - Linkage map of the common bean based on genotypic information from 85 marker loci of 91 recombinant inbred lines. Marker loci are listed on the right side of each linkage group and genetic distances (cM) are indicated on the left. "D", "Bng" and "P" markers are RFLP developed by Nodari *et al*. (1993a), Vallejos *et al*. (1992) and Adam-Blondon *et al*. (1994), respectively. "RoA" corresponds to RAPD markers from Nodari *et al*. (1993a). "Ro" refers to RAPD markers and "Sc" to SCAR markers described by Adam-Blondon *et al*. (1994). The isoenzyme markers *Chs* (chalcone synthetase), *Rbcs* (rubisco), *Diap-1* (diaphorase), *Aco-2* (aconitase), *Phs* (phaseolin) and *Chi* (chalcone isomerase) were described by Nodari *et al*. (1993a). NN_A and NN_B denote markers showing a significant association with NN after inoculation with *Rhizobium tropici* in the absence and presence of nitrogen, respectively. DLA_A and DLA_B denote markers significantly associated with DLA after inoculation with *X. axonopodis* pv. *phaseoli* in the absence and presence of nitrogen, respectively.

in the absence of nitrogen were not detected in its presence. These findings agree with published data, since mineral nitrogen is known to suppress the expression of some key proteins of the biological fixation pathway but has no effect on others. In the nitrate form, nitrogen inactivates the nodule-specific protein leghemoglobin and increases the number of unsuccessful infections, whereas in the ammonia form it inhibits the expression of nitrogenase but has no effect on glutamine synthetase genes in roots and nodules (Streeter, 1988; Cock *et al*., 1990; Franco and Neves, 1992). For the moment, however, we cannot establish any relationship between these two QTL and the above enzymes. Disease resistance QTL were also sensitive to nitrogen levels. The effects of QTL from LG 3, 7, and 9 were not seen in the presence of nitrogen, even though the first two QTL had the largest R^2 values in trial A. Conversely, the QTL from LG 2 was not detected in the absence of N. There are several well-established cases where the form of nitrogen influences host-pathogen interactions. In some pathosystems, ammonium enhances host susceptibility and nitrate has the opposite effect, whereas in other systems the opposite is true (Agrios, 1997).

Interestingly, the phenotypic effects of parental alleles at NN QTL on LG 3, 5, and 11 depended on the level of nitrogen. Thus, alleles from Jalo EEP558 at the QTL in LG 3 and 11 increased the number of nodules when no nitrogen was supplied, but otherwise generally decreased; the opposite was true for the QTL in LG 5. Considering that the experiments were conducted at the same time in the same greenhouse, it is plausible that this differential interaction reflected the effects of nitrogen fertilization. Although no differential interaction as described above for NN was no-

ticed for resistance QTL alleles, in two cases (LG 2 and 3) parental alleles behaved differently than expected. The occurrence of parental alleles with effects opposite to those predicted by their phenotype is well-documented (Young, 1996) and provides an explanation for transgressive segregants in progeny which could account for the occurrence of this phenomenon in this study. Such "cryptic" alleles (Thoday, 1961) could be used to develop superior genotypes.

Some markers showed a significant association with both traits in linkage groups 2, 3, 7, 10 and 11, reinforcing the idea of Nodari *et al*. (1993b) that there might be a set of genes that controls the interactions of the common bean with these two bacteria. The latter authors also observed the same association as noted above for markers in LG 7 using an $F_{2:3}$ population from which the RILs used in our study were derived.

The RILs used in this study were also used by Boscariol *et al*. (1998) to investigate the specificity of QTL to two strains of *Xanthomonas*, one of which was the same as that used in this study. Their results were comparable to the ones described here and provide more information on the stability of QTL for CBB resistance in different environments. QTL described here mapped to the same linkage groups as those reported by Boscariol *et al*. (1998), with several cases (8 out of 17) of significant marker-trait associations in both studies.

Dramatic differential QTL vs. environment interactions have never been demonstrated in the *P. vulgaris-R. tropici* symbiotic system. Our results stress the importance of considering the source and amount of nitrogen when breeding to improve the capacity to fix this element (Phillips and Teuber, 1992). However, in some cases the expression of certain NN and disease resistance QTL was not adversely affected by the nitrogen status. The latter QTL are suited to a breeding program aimed at developing cultivars adapted to variable soil fertility.

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RESUMO

Marcadores moleculares foram utilizados para estudar os efeitos do nitrogênio mineral na expressão fenotípica de QTLs associados ao número de nódulos (NN) e à resistência a *Xanthomonas axonopodis* pv. *phaseoli* em feijoeiro. Linhagens recombinantes obtidas do cruzamento BAT-93 x Jalo EEP558 foram avaliadas em casa-de-vegetação, sob dois níveis de adubação nitrogenada (0 e 5 mM de NH_4NO_3). A resistência ao patógeno foi avaliada pela medição da área foliar lesionada (DLA) e o NN por meio de contagem direta. Análises de variância foram empregadas para detectar associações significativas entre características quantitativas e genótipos das linhagens em 85 loci marcadores distribuídos em 12 grupos de ligação (LG). Sob a condição de ausência de N mineral, foram encontradas associações significativas entre 15 marcadores distribuídos em 7 LG para NN e 11 marcadores em 5 LG para DLA, explicando 34 e 42% da variação fenotípica destas características, respectivamente. Na presença de N, foram detectadas somente cinco associações significativas para NN e oito para DLA, explicando 28 e 26% da variação fenotípica de cada respectiva característica. Alguns QTLs foram detectados somente na ausência de N, evidenciando o efeito deste elemento na expressão destes QTLs. Entretanto, em alguns QTLs associados a NN, a contribuição dos alelos parentais foi dependente da concentração de N utilizada. Quatro QTLs foram encontrados associados tanto a número de nódulos como resistência a *Xanthomonas*, sugerindo um controle genético comum do feijoeiro à infecções bacterianas. Por outro lado, a expressão fenotípica de alguns QTLs não foi significativamente afetada pelo nível de nitrogênio utilizado. A estabilidade destes QTLs é interessante do ponto de vista de um programa de melhoramento voltado para o desenvolvimento de cultivares adaptadas a condições variáveis de fertilidade de solo.

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