

QUANTITATIVE TRAIT LOCI ASSOCIATED TO *Phytophthora parasitica* RESISTANCE ON CITRUS PLANTS

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ABSTRACT

Gummosis and root rot caused by *Phytophthora* spp. are among the most economically important diseases in *Citrus*, with *Phytophthora nicotianae* (*P. parasitica* Dastur) as the most frequent specie infecting *Citrus* in Brazil. This study was conducted to identify quantitative trait loci (QTLs) contributing to resistance to *P. parasitica* Dastur in a population of 80 F₁ hybrids derived from a cross between *Citrus sunki* x *Poncirus trifoliata* 'Rubidoux', respectively susceptible and resistant to the infection. Lesion size caused by *Phytophthora* 40 days after inoculation, observed in three consecutive years/environments (2001, 2002 and 2003), was used in the fitting of a mixed-effects linear model with terms for genotype, environment, and genotype-by-environment interaction factors. Broad sense heritability estimates on the basis of three years joint analysis was 14% ($P \leq 0.01$). Phenotypic means estimated under this model were used for QTL mapping analysis. As result, we found three QTLs associated with *Phytophthora* resistance in the linkage group II positions 8.4 cM and 77.7 cM (Phy2a and Phy2b) and linkage group IV position 33.6 cM (Phy4). Although we have found some punctual evidences of interaction, globally the LOD profile has changed only slightly suggesting that these evidences were in fact noise caused by model-overfitting. In the linkage group II, the two QTLs (Phy2a and Phy2b) explain 5.94% and 10.42% of the phenotypic variation, respectively, while the single QTL from linkage group IV (Phy4) explains 5.45% of the phenotypic variation. The QTLs Phy2a and Phy4 show negative additive effects, represented by smaller lesions on heterozygous plants at those predicted loci, while Phy2b shows positive additive effect, represented by larger lesions on heterozygous plants at that predicted locus. The results suggest that assisted selection of markers associated with QTLs could be used to enhance resistance to *Phytophthora* and may facilitate the breeding of resistant hybrid *Citrus* plants.

Keywords: *Poncirus trifoliata*, *Citrus sunki*, *Phytophthora parasitica*, QTL mapping,

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INTRODUCTION

Gummosis and root rot caused by *Phytophthora* spp. are among the most economically important diseases in *Citrus*, occurring in nearly all producing regions (Leoni and Ghini 2006). *Phytophthora nicotianae* Breda de Haan (*P. parasitica* Dastur) is the most frequent specie infecting *Citrus* in Brazil (Mourão et al. 2008). This soil-borne pathogen causes a multicyclic disease, living on both dead (necrotroph) and live (biotroph) plants, and reproducing both sexually and asexually (Bonnet et al. 2007). This pathogen infects the main scaffold branches of the tree inducing the formation of cankers with gum exudations, giving the branches a bleeding appearance. The expansion of the lesions upwards affects secondary branches, while downwards it affects the trunk (Alvarez et al. 2009).

Chemical control, such as metalaxyl and fosetyl Al, has been successfully applied both to gummosis and root rot, but its use is not always desirable because of the high cost of application, potential hazards to the environment, and the development of fungicide-resistant strains (Queiroz and Melo 2006). For these reasons, increasing the natural defense or resistance mechanisms of plants may represent an attractive strategy for preventing this disease (Del Rio et al. 2004). Few genetic studies using a controlled progeny have been performed to understand the inheritance mechanisms regarding resistance of *Citrus* to *Phytophthora*. According to Siviero et al. (2006), the study of the mode of inheritance of a disease resistance and other important traits in *Citrus* are hindered by factors like large plant size, long juvenility periods, self- and cross-incompatibility, inbreeding depression, and apomixis. These authors, while studying the mode of inheritance associated to the resistance against *Phytophthora* gummosis in *Poncirus trifoliata* identified three quantitative trait loci associated to the resistance, emphasizing the importance of the quantitative pattern of the manifestation of the disease. The assessment of resistance to gummosis in *Citrus* seedlings can be achieved by several inoculation methods. According to Siviero et al. (2002) the disc method and insertion of disc under the bark were the best inoculation methods for young plants under both orchard and greenhouse conditions. The best variable for disease assessment had been the measurement of the total lesion area. Lesion length can also be used when carried out in nurseries and in the field.

According to Chen et al. (2008) the *Poncirus* genera is a valuable resource in research as it possesses genes conferring many agriculturally important traits not

found in *Citrus*, which includes those associated with resistance to *Phytophthora*. Because of the valuable traits within some of the related genera that are absent from *Citrus*, many of the genetic as well as physical mapping projects have focused on *P. trifoliata* through intergeneric hybrids with *Citrus*. According to Talon and Gmitter Jr (2008), it is through the category of trait specific mapping that some of the promise of genomic science for *Citrus* genetic improvement is being pursued and realized, including selection of disease resistant and environmental stress tolerant hybrids in rootstock breeding programs, and targeted gene cloning projects aimed at providing potential solutions to serious disease problems.

The advent of molecular marker techniques has resulted in the construction of molecular maps to facilitate the study of quantitatively inherited traits (Shen et al. 2006). Their construction is highly valuable since it permits the location of markers flanking agronomically important genes, which can be useful in breeding programs for providing a better understanding of the genetic architecture of complex traits both within and between species (Zhang et al. 2004). Moreover, the use of molecular markers may result in early selection of genotypes with traits that could be expressed (and selected) only in adult plants (Tanksley et al. 1989). According to Jiang et al. (2007), QTL analysis on more extensive sources of resistance, especially novel improved germplasms, is of great importance and significance for accelerating the development of resistant genotypes.

Many studies have shown that the environment has a great influence on the phenotypic evaluation of certain characteristics, especially on quantitative traits (Lan et al. 2009, Dedryver et al. 2009; Rygulla et al. 2008). This implies that some genes have different effects according to the environment. These environmentally dependent gene effects can be of physiologic interest and the detection of such genes might have practical consequences for the breeding programs (Lillehammer et al. 2008). According to Boer et al. (2007), the incidence of genotype-by-environment interactions for quantitative traits has important implications for any attempt to understand the genetic architecture of these traits by mapping quantitative trait loci.

According to this proposal, in which the detection of environmentally dependent genes may increase the understanding of the biology behind genotype-by-environment interaction, linkage maps for *Poncirus trifoliata* (L.) Raf. Cv. 'Rubidoux'

has been developed exploiting the pseudo-testcross mapping strategy and RAPD markers (Cristofani-Yaly et al. 1999; Siviero et al. 2006), and were used to investigate QTLs associated to resistance to *Phytophthora* in a F₁ population. Plants were observed during three consecutive years, totalizing three time point evaluations of the trait in F₁ hybrids. Since the resistance was expected to be quantitative in nature, this strategy was used to enable more reliable identification of QTL over different environments and associated with the resistance profile.

MATERIALS AND METHODS

Plant material and *Phytophthora* inoculation.

Eighty hybrids derived from a cross between *Citrus sunki* Hort. ex. Tan. x *Poncirus trifoliata* (L.) Raf cv. 'Rubidoux' were selected from 314 field-grown plants (F₁ population). The hybrids were multiplied using buds that were grafted onto 6-month-old Rangpur lime rootstocks for each year of evaluation.

For this study were used *Phytophthora parasitica* isolated from *Citrus* roots (isolate IAC95). After six month, five plants from each hybrid were inoculated with *P. parasitica* by the disc method. The inoculation was performed by placing a mycelial block onto the center of a cut made in the stem and covered with parafilm. All plants were kept in an uncontrolled greenhouse under natural temperature and light until the final evaluation, which was achieved at 40 days post inoculation, by the measurement of the lesion size. Two uninoculated wound plants from each hybrid were used as control.

Experimental design was completely randomized and each plant represented one replicate. The assays were performed during three consecutive years, totalizing three time points of inoculation and evaluations.

Phenotypic analyses

A mixed-effects linear model with terms for environment, genotype and genotype-by-environment interaction effects was fitted on the observed lesion size measurements caused for *Phytophthora* inoculation (five observations per plant/environment), where the relative phenotypic variation explained exclusively by the genotype was the estimated broad-sense heritability. All factors but the intercept were modeled as

random effects. Calculations used the linear mixed effects “lmer” function in the “lme4” package to R (Bates and Sarkar 2007), and model parameters were obtained by replacing the respective parameters with restricted maximum-likelihood (REML) estimates. These estimators are best linear unbiased, so phenotypic means based on them are the BLUPs (Best Linear Unbiased Predictions) under this model and were used as input data for QTL mapping analysis. The broad-sense heritability (Wu et al. 2007) was defined as the ratio of the genotype variance over the phenotypic variance, i.e., $h^2 = \sigma^2_G / \sigma^2_G + \sigma^2_E + \sigma^2_{G \times E}$, where, h^2 =heritability, σ^2_G = variance of the genetic component, σ^2_E = variance of the environment component and $\sigma^2_{G \times E}$ = variance of the interaction between genes and environment. Standard error for h^2 was estimated using a leave-one-out jackknife procedure (Efron and Tibshirani 1993).

Molecular marker map

For this study, we used the linkage maps previously constructed from *C. sunki* and *P. trifoliata* by using a phase-unknown backcross model, where the strategy used in the mapping was a ‘pseudo-testcross’, based on selection of single-dose polymorphic markers present in one parent and absent in other (Cristofani et al. 1999). These linkage maps were based on the linkage analysis, 123 out of the 168 RAPD markers were grouped into 18 linkage groups. Sixty-three markers were linked to 10 linkage groups in *C. sunki* and in *P. trifoliata* cv. ‘Rubidoux’, 60 markers were found to be linked to 8 linkage groups. The total length of the maps was 867.58 cM for ‘Rubidoux’ and 732.32 cM for *Sunki*, with an average distance of 14.45 cM between markers for ‘Rubidoux’ and 11.62 cM for *Sunki*. The individual length of the linkage groups shown a variation between 3.20 and 246.67 cM for ‘Rubidoux’ and 10.3 and 174.18 cM for *Sunki*. The highest distance between markers was 43.8 cM. By using a distance higher than 30 cM as a gap determination criterion, three gaps were found in *Sunki* and six gaps in ‘Rubidoux’. The linkage maps were already used in assays that focused on locating genes for resistance against the *Citrus tristeza virus* (CTV) (Cristofani et al. 1999) and mapping of resistance against *Phytophthora* gummosis (Siviero et al. 2006).

QTL Linkage Analysis

Dominance effects of each QTL were calculated using phenotypic means estimated by the systematic component of the fitted mixed-effects linear model used in broad-sense heritability calculation using a genomewide one-dimensional scan of the *P. trifoliata* map with the Haley–Knott regression algorithm implemented in the “scanone” function in the R/qtl program v.1.14-2 (Broman et al. 2003), with walkspeed of 2.1 cM as calculated by the SRmapQTL routine of the QTL Cartographer (QTL Cart) v.1.16 (Basten et al. 2002). Linkage analysis results were expressed as LOD scores. Genomewide LOD significance thresholds were estimated after permutation tests that had been replicated 4,000 times under the null hypothesis that there were no QTL anywhere in the genome (Churchill and Doerge 1994). Four confidence thresholds were calculated at 1, 5, 10, and 20% significance levels. QTL with LOD scores > 1% were considered highly significant, those > 5% were considered significant, and those > 10% were considered suggestive (Lander and Kruglyak 1995). For initial QTL identification, a genomewide one-dimensional scan a model considering only QTL as a factor was fitted. Estimation of percentage of variance explained by each QTL was obtained from a drop-one-term analysis of results in an additive model. The additive model was calculated using the “fitqtl” function with imputation method in the R/qtl program (Sen and Churchill 2001). The 95% confidence intervals for the chromosomal positions of putative QTL were approximated by the Bayesian credible intervals (Broman and Sen 2009).

RESULTS

Evaluation of the inoculation of *P. parasitica*

The two parents, *C. sunki* and *P. trifoliata*, consistently displayed significant differences in response to *Phytophthora* infection in the three consecutive years. As expected, *P. trifoliata* had a higher level of resistance while *C. sunki* had a higher level of susceptibility. Averaged over the three years, mean of the lesion size of *P. trifoliata* and *C. sunki* was 6.46 ± 0.92 and 16.06 ± 2.04 mm, respectively. Overall mean of the lesion size of the F₁ hybrid in the three experiments was 10.76 ± 3.13 . In year 2001, the mean of the lesion size of the parents was 5.4 ± 2.07 and 18.4 ± 2.3 mm for *P. trifoliata* and *C. sunki*, respectively, and the lesion size for the F₁ individuals ranged from 7.6 to 18.2 mm (11.55 ± 3.4). In year 2002, the parents *C.*

sunki and *P. trifoliata* 'Rubidoux' shown the means 14.6 ± 2.7 and 7.0 ± 1.0 mm, respectively and the lesion size for the F₁ individuals ranged from 5.8 to 17.2 mm (10.4 ± 3.3). In year 2003, the parents *C. sunki* and *P. trifoliata* 'Rubidoux' shown the means 15.2 ± 2.39 and 7.0 ± 0.71 mm, respectively and the lesion size for the F₁ individuals ranged from 6.4 to 16.8 mm (10.35 ± 2.5). The lesion length coefficient of variation from F₁ hybrids was 29.46%, 31.71%, 23.84% and 29.08% in the years 2001, 2002 and 2003 and over all three years (joint analysis), respectively (Table 1).

Table 1. Mean \pm standard deviation of lesion longitudinal length mean, in millimeters, from the parent and hybrid populations in the three time point evaluation after *P. parasitica* inoculation.

| Experiments | Parents | | Population F ₁ | | |
|----------------|----------------------|----------------|---------------------------|------------|-------|
| | <i>P. trifoliata</i> | <i>Sunki</i> | Mean Lesion | Range | CV% |
| 2001 | 5.4 ± 2.07 | 18.4 ± 2.3 | 11.5 ± 3.4 | [7.6;18.2] | 29.46 |
| 2002 | 7.0 ± 1 | 14.6 ± 2.7 | 10.4 ± 3.3 | [5.8;17.2] | 31.71 |
| 2003 | 7.0 ± 0.71 | $15.2 \pm$ | 10.35 ± 2.5 | [6.4;16.8] | 23.84 |
| | | 2.39 | | | |
| Joint analysis | 6.46 ± 0.92 | $16.0 \pm$ | 10.76 ± 3.13 | [5.8;18.2] | 29.08 |
| | | 2.04 | | | |

The frequency distribution mean of the lesion size caused by *Phytophthora* inoculation of the F₁ population was approximately normal (data not showed) in all experiments, suggesting polygenic and quantitative inheritances of *P. parasitica* resistance. Means of the lesion across the three experiments, including the parental trait, ranged from 5.4 to 18.4 mm. No hybrid was more resistant than *P. trifoliata* 'Rubidoux' or more susceptible than *C. sunki* on the basis of the least significant difference ($P < 0.05$). However, there were 15 hybrids that shown similar resistance profile than that of the *P. trifoliata* 'Rubidoux' ($P < 0.05$) (data not showed).

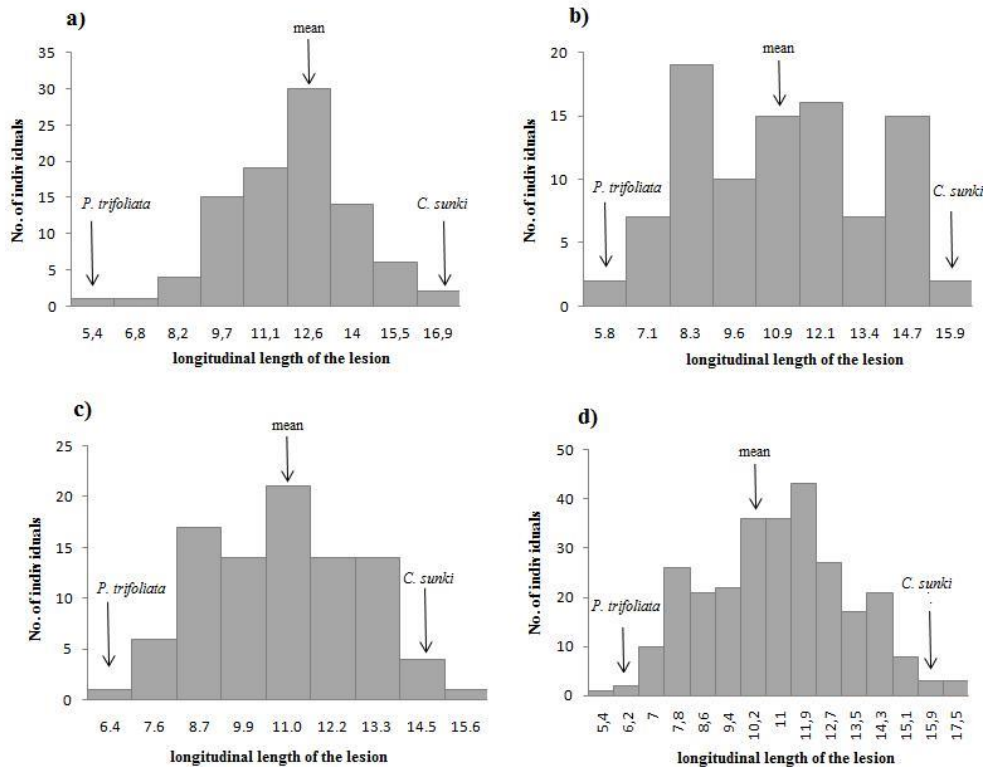


Figure 1. Frequency distribution of longitudinal length of the lesion, in millimeters, from the parents and hybrids in three different time point evaluation after *P. parasitica* inoculation. a) year 2001; b) year 2002; c) year 2003 and d) Overall. The values indicated on the x-axis are the lower limit of each category.

To assess the relative contributions of genotype and environment, broad-sense heritabilities and environment effects were estimated by mixed-effects linear model analysis of phenotypic data collected from the measurement of lesion size caused for *Phytophthora* inoculation in a F₁ population (*Citrus sunki* × *Poncirus trifoliata* ‘Rubidoux’) in three different years. An analysis of variance shown that genotype variation among F₁ population was significant in each year and in joint analysis ($P < 0.01$) (Table 2). Variation due to different environment (experiments) and genotype-by-environment interaction variance was also significant. The estimates of heritability in broad sense for single experiments 0.25 ± 0.05 , 0.51 ± 0.03 and 0.57 ± 0.03 in the years 2001, 2002 and 2003, respectively. Broad sense heritability estimates on the basis of three years joint analysis was 0.14 ± 0.02 (Table 2).

Table 2. Estimates of genetic parameters for resistance to *P. parasitica* in 91 recombinant F₁ lines derived from the cross between *C. sunki* and *P. trifoliata* 'Rubidoux' measured over 3 years.

| Environment | Parameters | | | |
|----------------|--------------|---------------------|--------------------------------|-------------------|
| | $h^2 \pm SE$ | $\sigma^2_G \pm SE$ | $\sigma^2_{G \times E} \pm SE$ | $\sigma^2 \pm SE$ |
| 2001 | 0.25 ± 0.05 | 3.14 ± 0.18* | - | 3.03 ± 0.14 |
| 2002 | 0.51 ± 0.03 | 5.68 ± 0.24* | - | 5.38 ± 0.10 |
| 2003 | 0.57 ± 0.03 | 3.66 ± 0.19* | - | 2.75 ± 0.07 |
| Joint analysis | 0.14 ± 0.02 | 1.51 ± 0.12* | 2.98 ± 0.10* | 5.77 ± 0.06 |

* indicates variance component is significant at the $P < 0.01$ level of probability.

h^2 =broad sense heritabilities

σ^2_G = genotypic variance

$\sigma^2_{G \times E}$ = genotype-by-environment interaction variance

σ^2 = error variance

Although we have encountered a strongly environment impact in the phenotypic outcome, correlation analysis have shown that only between years 2002 and 2003 we have positive correlation, 0.78 (CI95%=[0.69,0.85]), among phenotypics, which strongly suggests that hybrids faced different climate conditions in year 2001. This suspicion was confirmed by non-parametric analysis (Kruskal-Wallis test) that shown that the rank of resistance among hybrids were significantly different ($P \leq 0.05$) between years 2001 and 2002, 2001 and 2003, but not between years 2002 and 2003. Also, by year-by-year moderated T-tests between hybrids and parents *P. trifoliata* 'Rubidoux' or *Citrus sunki*, we have shown that while in year 2001 no hybrids was significantly (Holm adjusted $P^* \leq 0.05$) more resistant or susceptible than parents, in 2002, 39 hybrids were significantly resistant ($P^* \leq 0.05$) compared to *P. trifoliata* and 28 hybrids were significantly susceptible ($P^* \leq 0.05$) compared to parent *Citrus sunki*, and in 2003, 30 hybrids were resistant and no hybrids susceptible ($P^* < 0.05$). As expected, among the top 39 hybrids resistant in the year 2002, 26 of them were also resistant compared to *P. trifoliata* in the year 2003 while only 13 of them were exclusively resistant in 2002. Also, in the year 2003, among the top 30 hybrids resistant 4 of them were exclusively resistant in that year (Fig. 2).

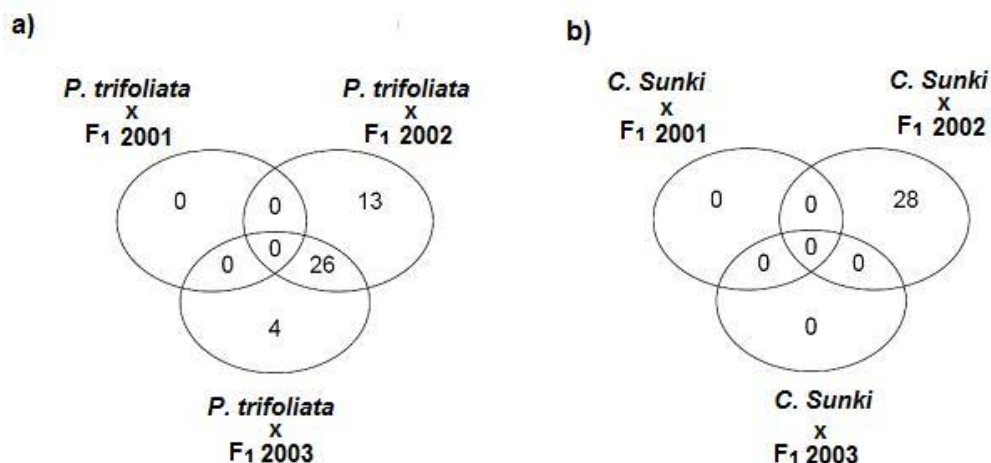


Figure 2. Venn diagrams showing the comparison among F₁ hybrids and their parents after inoculation with *Phytophthora parasitica* in three consecutive years. (a) *Poncirus trifoliata* 'Rubidoux' and (b) *C. sunki*, respectively resistant and susceptible to *P. parasitica*.

Estimation of QTLs associated to the resistance against *Phytophthora*

Given the evidence of the environment influence in observed phenotypic, QTLs were analyzed using interval mapping with Haley-Knott regression procedure with response data given by means of the lesion size caused by *Phytophthora* inoculation, as outputted by the fitted mixed-effects linear model used in broad-sense heritability estimation, and with environment, given by the three consecutive years, as covariate. As result, we found two QTLs associated with *Phytophthora* resistance. The most significant ($P \leq 0.01$) QTL was associated with the marker AV03_250 in the linkage group II, position 77.1 cM, with LOD score of 6.67, followed by the marker cc4.loc29.4 ($P = 0.06$) located in the linkage group IV, position 29.4 cM, with LOD score of 2.40. We also made another interval mapping removing the two most significant QTLs found to look for QTLs hindered by the effect of the most significant QTLs. We then found a third QTL associated with the marker cc2.loc8.4 ($P = 0.019$) located in the linkage group II, position 29.4 cM, with LOD score of 2.97. We then constructed a model with the three selected QTLs and environment as covariate, and found the linkage group II, positions 8.4 cM and 77.7 cM, and the linkage group IV, position 33.6 cM, as the most probable for the three QTLs (Fig. 3).

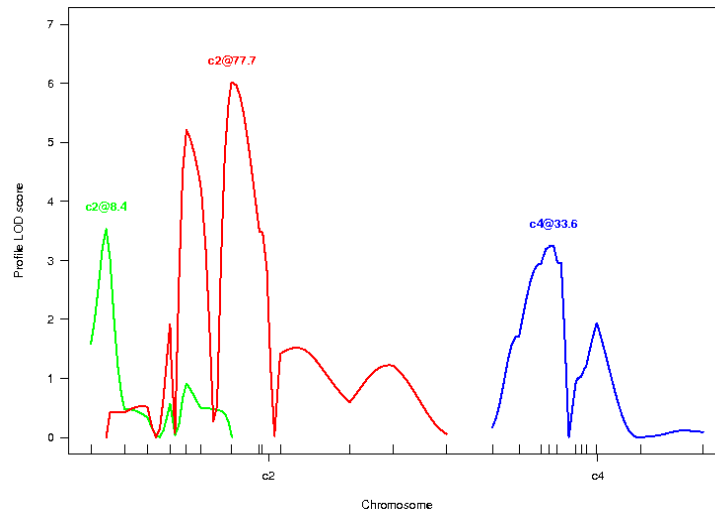


Figure 3. Profile LOD score curves for a three-QTL model for the *Phytophthora* resistance data. c2 and c4 indicate linkage groups II and IV, respectively.

We have also fitted models with QTL x QTL interactions and QTL x Environment. For the first one, we could not find evidence of interaction between QTLs on linkage group II, at 8.4 cM and 77.7 cM ($P = 0.34$), but found evidence of interaction between QTLs located on linkage group II and IV, at 77.7 cM and 33.6 cM ($P = 0.06$), and at 8.4 cM and 33.6 cM ($P < 0.01$). For the model augmented with QTL x Environment we could find evidence of interaction between the QTL located on linkage group II at 77.7 cM in year 2002 ($P = 0.06$), and the QTL located on linkage group II at 8.4 cM in years 2002 ($P = 0.01$) and 2003 ($P = 0.08$). Although we have found some punctual evidences of interaction, globally the LOD profile have change only slight (evidenced by the red flat lines in Fig. 4), suggesting that these evidences were in fact an overfitting artifact.

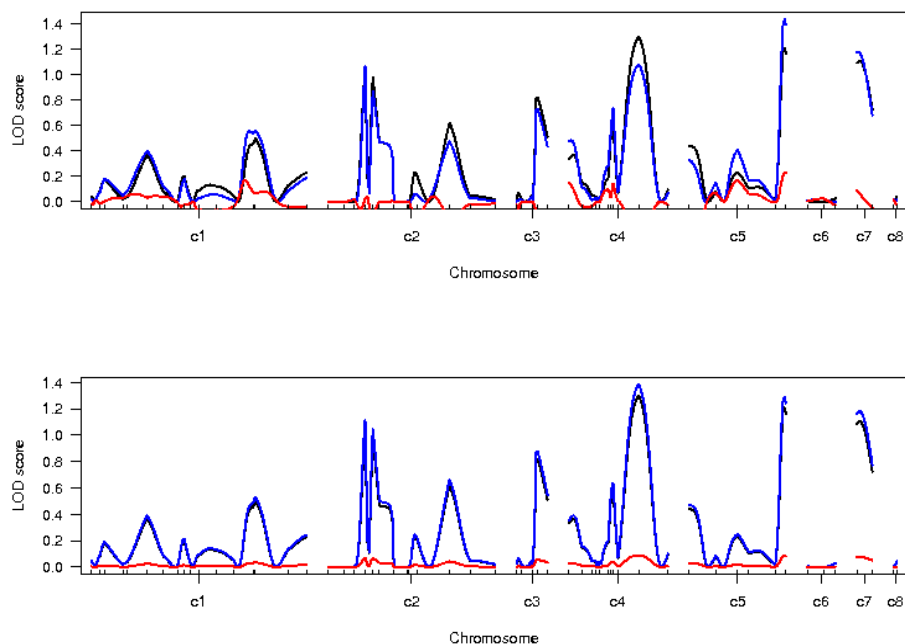


Figure 4. LOD score curves from genome scans by Haley-Knott regression for the *Phytophthora* resistance data models. Blue LOD curves indicate the model with environment as an additive covariate; Black LOD curves indicate model with environment as an additive covariate augmented with QTL x QTL interaction (top) and with QTL x Environment interaction (bottom). Red lines indicate the difference LOD between fitting the additive model (in blue) and augmented model (in black).

After choosing the additive model, with Environment as a additive covariate and no interaction of any kind, as the most parsimonious model to explain the *Phytophthora* resistance phenotypic observed, we have computed Bayesian credible intervals for the three QTLs in figure 3 (Table 3). In the linkage group II, the two QTLs (Phy2a and Phy2b) explain 5.94% and 10.42% of the phenotypic variation, respectively, while the single QTL from linkage group IV (Phy4) explains 5.45% of the phenotypic variation.

Table 3. Bayesian credible interval for the three QTLs associated with *Phytophthora* resistance.

| QTL | Link. Groups | Position (cM) | LOD score | Marker Interval | Interval (cM) | Phenotypic %var |
|-------|--------------|--------------------|-----------|---------------------------|------------------|-----------------|
| Phy2a | II | 8.4 (C12_1500) | 3.53 | cc2.loc4.2 cc2.loc10.5 | - 4.20 10.5 | - 5.94 |
| Phy2b | II | 77.7 (AV03_250) | 6.01 | AV16_140 cc2.loc86.1 | - 52.46 86.10 | - 10.42 |
| Phy4 | IV | 33.6 (U11_1550) | 3.25 | cc4.loc16.8 U03_1550 | - 16.80 57.35 | - 5.45 |

To better understand the QTLs effect on the observed phenotypic, we found the nearest markers of the inferred QTLs, C12_1500, AV03_250 and U11_1550, for Phy2a, Phy2b and Phy4, respectively, and displayed the phenotypics of all hybrid plants according to the genotypes at these closest markers (Fig. 5). We can see that there is a slightly increase in lesion size in heterozygous plants at the C12_1500 marker, while there is a decrease in lesion size for heterozygous plants at both AV03_250 and U11_1550 markers. We can say that the QTLs Phy2a and Phy4 show negative additive effects and Phy2b shows positive additive effect in the mean length of the lesion.

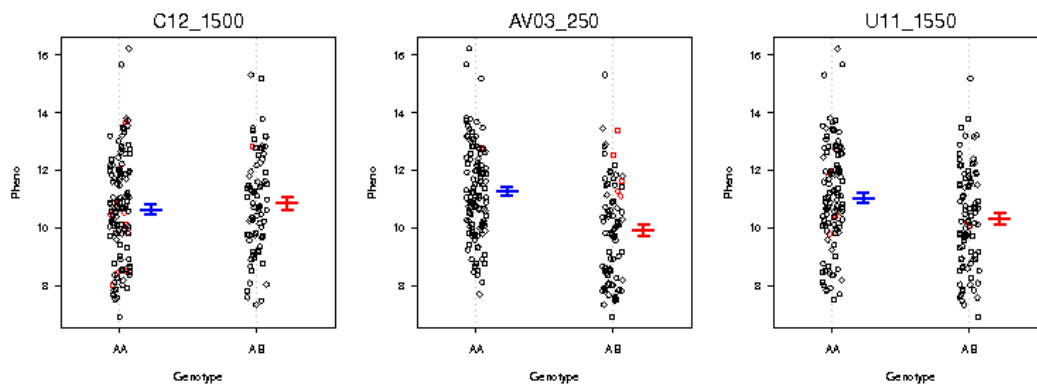


Figure 5. The effect of putative QTLs on the *Phytophthora gummosis* resistance phenotypic. Left: a dot plot of the phenotypic as a function of marker genotypes, with black dots corresponding to observed genotypes and red dots corresponding to missing (and so imputed) genotypes.

Finally, we investigated if there was an epistatic effect between QTLs Phy2a and Phy2b, as they were in the same linkage group. As can be seen in figure 6, the two recombinant classes (haplotypes AA/AB or AB/AA at AV03_250 and C12_1500, respectively) have a slightly different phenotypic average than non-recombinant: plants that are AA/AB have lesion sizes slightly larger than AA/AA, while plants that are AB/AA have lesion sizes slightly shorter than AB/AB individuals. The same feature is seen in the output of the effect plot: the two QTLs appear to have effects of opposite sign, but approximately the same magnitude.

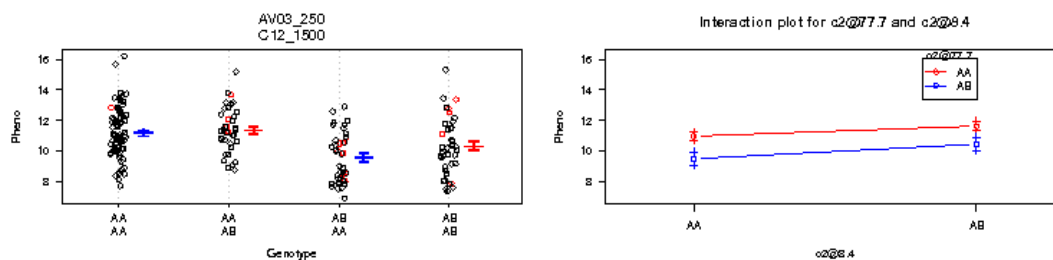


Figure 6. The effect of two putative linked QTL on chromosome 2 on the *Phytophthora gummosis* resistance phenotypic. Left: a dot plot of the phenotypic as a function of marker genotypes, with black dots corresponding to observed genotypes and red dots corresponding to missing (and so imputed) genotypes. Right: estimated phenotypic averages to each of the four two-locus genotype groups, at the inferred locations of the two putative QTL.

DISCUSSION

The F₁ population derived from a cross between *Citrus sunki* Hort. ex. Tan. and *Poncirus trifoliata* (L.) Raf cv. 'Rubidoux' was initially developed for mapping genetic loci for resistance against the *Citrus tristeza virus* (CTV) (Cristofani et al. 1999) and mapping of resistance against *Phytophthora gummosis* (Siviero et al. 2006). This population was a good choice for mapping disease resistance because the *Poncirus* genera possesses genes conferring many agriculturally important traits not found in *Citrus*, including genes responsible for resistance to *Phytophthora* (Chen et al. 2008). In *Citrus* mapping, most of the populations that have been used worldwide to develop genetic maps for genomes and QTLs were derived from crosses with *Poncirus*, because of the high interest in its exclusive genes (Siviero et al. 2006; Ruiz and Asins 2003; Cristofani et al. 1999).

In the present study, phenotypic data collected in three consecutive years (2001 to 2003), represented by the measurement of the lesion longitudinal size caused by *Phytophthora* inoculation, shown an approximately normal distribution in each year and in the mean of the lesion size of the three consecutive years (joint analysis), which strongly suggests that inheritances of *P. parasitica* resistance is polygenic and quantitative. Similar phenotypic distributions for the resistance against *Phytophthora gummosis* were observed by Siviero et al. (2006). According to Thabuis et al. (2004),

quantitative and polygenic resistance, also characterized and exploited in plant breeding, can confer an efficient control of disease severity. Quantitative resistance is of high interest in disease management strategies due its durability in the field (Ayme 2005).

The suggestion of polygenic control is consistent with specialized associations that may exist between hemibiotrophic pathogens such as *P. parasitica* and the host *Poncirus*, which, as expected, had a high level of resistance relative to *C. sunki* in all years observed. Biotrophic and hemibiotrophic pathogens invade living cells and subvert metabolism to favor their growth and reproduction; hence minor differences in either organism can upset the balance (Takemoto et al. 2005). In the present study, an analysis of variance with the F₁ population shown that environment differences among consecutive years were significant ($P < 0.01$) to explain observed variance in the trait (Table 2). No hybrid was more resistant than *P. trifoliata* 'Rubidoux' or more susceptible than *C. sunki* ($P < 0.05$). However, there were 15 hybrids that shown similar resistance of that observed in the *P. trifoliata* 'Rubidoux' ($P < 0.05$). Overall, the contrasting reaction of resistance and susceptibility observed in the F₁ population in all years suggest some degree of specificity governed by not one, but a group of genes in the resistance of *P. trifoliata* to *P. parasitica*.

In addition, the estimates of heritability in broad sense for single experiments were 0.25, 0.51 and 0.57 in 2001, 2002 and 2003, respectively. The heritability in 2001 was lower than the others possible because the environment was more favorable for the parasitic infection severity, which is suggested for the higher mean observed both in *Citrus sunki* susceptible parents (18.4 ± 2.3), and in the overall hybrid F₁ population (11.55 ± 3.4), reflected in a lower contrasting reaction between resistance and susceptibility. Consequently, no hybrids could be distinguished as resistant or susceptible ($P < 0.05$) when compared to parents *P. trifoliata* 'Rubidoux' or *Citrus sunki*, respectively. Broad sense heritability estimate on the basis of three years combined analysis was 0.14. The low broad-sense heritability estimated in the present study is in agreement with Siviero et al. (2006), which found low level of heritability (0.18). Low heritability is common in quantitative traits due to the importance of the environmental effect on the behavior of the trait being studied (Waaij et al. 2006). This result emphasizes the importance of multi-experiment data

for this trait. Since quantitative traits are strongly influenced by the environment, it is essential to repeat testing in subsequent years or different locations to distinguish the environment component of variation from the genetic one.

We found three QTLs associated with *Phytophthora* resistance in the linkage group II positions 8.4 cM and 77.7 cM (Phy2a and Phy2b) and linkage group IV position 33.6 cM (Phy4). For QTL x QTL interactions, we could not find evidence of interaction between QTLs Phy2a and Phy2b, but found evidence of interaction QTLs Phy2a and Phy4 and between Phy2b and Phy4.

As describe above, the assays were performed during three consecutive years using six-months-old plants, totalizing three time point inoculation and evaluations in an uncontrolled greenhouse under natural temperature and light. For the QTL x Environment model we could find evidence of interaction between the QTL Phy2b in year 2002 ($P=0.06$), and the QTL Phy2a in years 2002 ($P=0.01$) and 2003 ($P=0.08$). Although we have found some punctual evidences of interaction, globally the LOD profile have change only slightly, suggesting that these evidences were in fact an overfitting artifact. Recent developments in QTL analyses have provided detailed information about possible loci that may perform differently under different environmental conditions, by comparing the QTLs that were detected in multiple environments (Van Eeuwijk et al. 2010; Cooper et al. 2009). According to Lan et al. (2009), genotype-environment interaction is an important component especially affecting quantitative traits. A QTL detected at a specific map region in one environment but not in another may indicate QTL by environment interaction. In the absence of a true QTL and environment interaction, however, a QTL can be detected in one environment and not in another environment because the probability of simultaneous detection in both environments is strongly reduced (Benmoussa et al. 2006).

In the linkage group II, the two QTLs (Phy2a and Phy2b) explain 5.94% and 10.42% of the phenotypic variation, respectively, while the single QTL from linkage group IV (Phy4) explains 5.45% of the phenotypic variation. The phenotypic variance explained (R^2) by all QTL for all years were low. Comparison with the values of broad-sense heritabilities suggested that all the genetic variance was not explained

by these QTL. Heritability is the proportion of phenotypic variation in a population that is attributed to the genetic variation among individuals (Falconer and Mackay, 1996). Heritability analysis estimates the relative contributions of genetic and non-genetic factors to the total phenotypic variation in a population trial. This may result from the existence of resistance loci in genomic regions not covered by our genetic map or from the choice of the significance threshold which could have prevented the detection of QTL.

Heterozygotes are expected to have phenotypic values corresponding to the mean of the parental values; deviation from the mean indicates a dominant effect at or near that marker. The sign of the effect indicates the direction of change in the phenotypic; in this study, a positive sign indicates an increase and a negative sign a decrease in the trait value, which the QTLs Phy2a and Phy4 show negative additive effects and Phy2b shows positive additive effect in the mean length of the lesion. According to Doust et al. (2005) QTL with additive effects of differing sign for a particular trait indicate that each parent contains a mixture of alleles for that trait, some acting to change the phenotypic toward that of one of the parents while others act to change the phenotypic toward the other parent.

Finally, we investigated if there was an epistatic effect between QTLs Phy2a and Phy2b, as they were in the same linkage group. As can be seen in figure 6, the two recombinant classes (haplotypes AA/AB or AB/AA at AV03_250 and C12_1500, respectively) have a slightly different phenotypic average than non-recombinant: plants that are AA/AB have lesion sizes slightly larger than AA/AA, while plants that are AB/AA have lesion sizes slightly shorter than AB/AB individuals. The same feature is seen in the output of the effect plot: the two QTLs appear to have effects of opposite sign, but approximately the same magnitude. This result suggests that the two QTL are linked in repulsion, and so the region should exhibit little marginal effect (what can be seen by the phenotypic percent variation explained by any of them), but when both loci are considered, the two may stand out clearly. On the other hand, the two linked loci on linkage group II do not show evidence of epistatic interactions (effect plot shows parallel lines), and so must segregate independently.

This was the first time that stability of QTLs associated to *Citrus* Gummosis caused by *Phytophthora* was studied. The results suggest that markers associated with QTLs could be used in marker-assisted selection to enhance resistance to *Phytophthora* and may facilitate the breeding of resistant *Citrus* by marker assisted selection. The consistency of QTLs across different years of evaluation suggest the presence of genes that were stables in different environment and could be useful in breeding programs or orchard management decisions.

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