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Original article

Genetic variation of *Prunus avium* in susceptibility to cherry leaf spot (*Blumeriella jaapii*) in spatially heterogeneous infected seed orchards

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Abstract – Cherry leaf spot (*Blumeriella jaapii* (Rehm) Arx.) is a very serious disease of wild cherry (*Prunus avium* L.), which produces premature leaf defoliation and vigor decrease. In two clonal seed orchards of *P. avium* naturally infected by *B. jaapii*, spatial heterogeneity and autocorrelation of neighbor damage caused by cherry leaf spot impeded proper analysis of the fungus incidence. The iterative spatial analysis (ISA), based on variography and kriging, was successfully used to eliminate the effect of this spatial heterogeneity in analysis of genetic variation in susceptibility to *B. jaapii*. Significant differences among *P. avium* clones were found, with moderate to high broad-sense heritability estimates. Genetic by environment interactions, although significant, were not quantitatively important. A strong relationship between leaf spot susceptibility and bud burst was found. However, other factors must be affecting the genetic variation in leaf spot susceptibility, as differences among clones remained highly significant when considering the bud burst as a covariate in the genetic model.

wild cherry / genetic resistance / bud burst / broad-sense heritability / spatial distribution

Résumé – Variabilité génétique de la susceptibilité de *Prunus avium* à *Blumeriella jaapii* dans des vergers à graines infectés spatialement de manière hétérogène. *Blumeriella jaapii* (Rehm) Arx. est une des causes principales du dépérissement du merisier (*Prunus avium* L.) : il produit une défoliation prématurée et réduit la vigueur des arbres. Dans deux vergers à graines de clones de merisier naturellement infectés par *B. jaapii*, l'hétérogénité spatiale des dégâts et l'autocorrélation entre voisins empêchent une analyse correcte de l'incidence du champignon. Afin d'éliminer ces effets et d'étudier correctement la variabilité génétique de la susceptibilité au champignon, on a utilisé avec succès l'analyse itérative spatiale (ISA), basée sur la variographie et le krigeage. Des différences significatives entre clones existent pour la sensibilité au champignon. L'héritabilité clonale pour ce caractère est modérée à forte. Bien que l'interaction clone × site soit significative, elle n'est pas quantitativement importante. Par ailleurs, on a trouvé une relation étroite entre la susceptibilité au champignon et le débourrement végétatif. Néanmoins, d'autres facteurs doivent affecter la variabilité génétique à la susceptibilité, car les différences entre clones restent très significatives même quand on utilise le débourrement comme covariable dans le modèle statistique.

merisier / résistance génétique / débourrement / héritabilité / distribution spatiale

1. INTRODUCTION

Cherry leaf spot is a very serious *Prunus* sp. disease caused by the fungus *Blumeriella jaapii* (Rehm) Arx. [10, 15, 30], affecting, among other species, the commercially important forest tree wild cherry (*Prunus avium* L.) [1–3, 19]. In fact, it has been recognized as the worst sanitary problem of wild cherry in some European countries [26]. Cherry leaf spot produces premature leaf defoliation, vigour decrease, especially in diameter, and winter hardiness reduction, which can even induce tree death due to low winter temperatures [33].

The cherry leaf spot fungus is spread by two different kinds of spores [11]. The fungus overwinters in fallen diseased leaves on the ground, which were colonized during the previous growing season [32]. In spring, the fruiting structures (apothecia) develop and, during wet periods, release ascospores. These primary windblown spores land on new enlarging leaves where the fungus penetrates through stomata. Infection occurs during moist periods when leaves remain wet. This first infection is limited, not only because the new leaves are small and not as susceptible, but also because the stomata of these leaves are still immature. This mode of infection suggests that leaf phenology could play a key role in the infection process, and thus, screening for resistance to this disease should consider this possible influence. After infection of the current year's leaves, acervuli are formed on their underside producing conidia, which are responsible for the extensive spread of the disease [11]. These secondary spores are rain splashed to neighbor foliage where germinate to enable additional infection [15]. If weather conditions for disease development are conductive, infection can become increasingly abundant as the season progresses. New infections can occur throughout the summer and fall due to the rapid increase and spread of the fungus during wet periods by means of repeated generations of conidia.

Wild cherry is one of the most valued European forest tree species and is considered a noble hardwood. Its wood is mainly used for panelling and cabinet-making and achieves

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really high prices in the wood market. Due to its valuable wood and its fast growth (rotation of 50–60 years), the species is now increasingly planted in Europe, both in afforestation of abandoned farmland and enrichment of forests [26]. In Galicia (NW Spain) a long term breeding program for this species was started in the 90's looking forward to improve timber quality and production. This breeding program has included phenotypic mass selection and the use of this material for seed production in clonal seed orchards. The natural infection by *B. jaapii* in two seed orchards gave us the opportunity to analyze the genetic variation in susceptibility to this fungus disease, and to explore the possibility to improve resistance through breeding. Given the importance of this disease, improving resistance could become a main breeding objective. In fact, breeding for resistance to leaf spot disease is a major goal in many cherry breeding programs [2, 20, 26, 30, 33]. Within these breeding programs, several authors have studied the genetic variation of *P. avium* to different diseases, including cherry leaf spot [2, 20, 26, 27]. Breeding for resistance to *B. jaapii* is possible as variation of resistance among genotypes has been shown in France among 33 clones [26] and among 14-parent half diallel [20], and in Belgium among 19 clones [2], with broad-sense heritability ranging between 0.56 and 0.96. Resistance to other diseases or pests, such as aphids or bacterial canker, is also heritable, but in less degree (0.40 for aphids [26] and 0.27–0.51 for bacterial canker [27]).

The development of fungus diseases usually follows an heterogeneous spatial structure with the probability of infection distributed in aggregates or gradients [14, 25, 31]. This spatially heterogeneity complicates the screening of genetic entries in field trials, as the autocorrelation of neighbour data implicates a violation of the ANOVA assumptions (e.g. [6, 9]). In order to properly analyze the genetic variation in susceptibility to any fungus disease, one should firstly explore the spatial structure of the disease incidence and, if nonrandom structures are detected, an appropriate method should be used to account for the spatial heterogeneity. Several methods are available to account for this spatial variation and, among them, geostatistics (see Material and methods) has shown promising results both in agriculture and forest experiments [9, 34].

The objectives of this paper were: (i) to explore the spatial structure of the disease incidence and to outline the importance of adjusting the data when spatial autocorrelation is present, (ii) to determinate the level of genetic variation in the susceptibility to cherry leaf spot in the Galician *P. avium* breeding population, and (iii) to determine the relationship between leaf spot susceptibility and bud burst, as this fungus penetrates by stomata.

2. MATERIAL AND METHODS

2.1. Material and sites

The studied sites are two *P. avium* clonal seed orchards located in Galicia (NW Spain): Areas (42° 01' N, 8° 40' W, 90 m a.s.l.) and Sergude (42° 49' N, 8° 27' W, 270 m a.s.l.). Both sites have an acidic soil (pH in water around 5.2 in both sites) above amphybolites in Sergude and granites in Areas. The climate at both seed orchards is Atlantic. Areas has a mean temperature of 14.2 °C and 2503 mm for total annual rainfall (observation period 1991–2002, Monte Aloia climatic station, 42° 04' N, 8° 40' W, 400 m a.s.l.), while Sergude has 13.8 °C for mean temperature and a total annual rainfall of 1574 mm (observation period 1991–2002, Sergude climatic station, 42° 49' N, 8° 27' W, 225 m a.s.l.). May temperature and rainfall are 15.0 °C and 204 mm at Areas and 14.6 °C and 158 mm at Sergude, respectively.

One hundred and fifty-five plus trees, selected for growth and form within natural stands from North Spain, were grafted and planted in these two seed orchards. One hundred and twenty-nine clones were installed in Areas in January 1998, whereas 80 clones were planted in Sergude in March 2002. Only 44 clones were in common in both seed orchards. Both seed orchards were installed with the aim to provide high genetic quality seeds for reforestation in Galicia. They follow a randomized complete block design. Areas has 129 clones, 7 blocks, one-tree plot and 3×3.4 m spacing, while Sergude has 80 clones, 10 blocks, one-tree plot and 5×5 m spacing.

2.2. Assessments

Cherry leaf spot disease was measured in May of years 2000, 2002, 2004, and 2005 in Areas and in year 2005 in Sergude. More than 96% of the trees were affected, in more or less extent, by this disease all years of study in each site. Intensity of the disease damage was subjectively determined using a six-level scale from 0 to 100% (of foliage damaged area), by 20%. Bud burst data was also assessed in March of years 2004 and 2005 at Areas and in 2005 at Sergude, following a 13-level scale, based on: bud elongation size, leaf differentiation and size, and shoot length (from 1 = closed bud to 13 = open leaves and annual growth over 5 cm). Both, cherry leaf spot and bud burst were assessed in a unique date each year. Only 122 clones at Areas and 71 clones at Sergude with complete data set were used for the genetic analysis of each separate site. The 44 common clones were used for the joint analyses of both sites.

Leaf samples were randomly collected from 10 diseased trees at each site and each year of study, and the pathogen identification was verified by isolation at the Areeiro Phytopathological Centre (Pontevedra, Spain).

2.3. Spatial Analysis

Infection of leaf spot disease, from fallen diseased leaves on the ground to new developing leaves of neighboring trees and from infected to uninfected foliage, suggests the possibility of spatial heterogeneity, which can implicate a violation of the assumption of residual independence of the analysis of variance. Residuals of each variable after subtracting the clone effects were used to explore for any spatial structure in the data. A one-way analysis of variance with the clone effect considered random was carried out, and the clonal breeding values (BLUP) were obtained using the MIXED procedure of the SAS system [28]. The spatial structure of the resulting residuals was analyzed using a semivariogram, which plots the semivariance between plots as a function of the distance separating them [21]. For randomly distributed data, little change in the semivariance will be obtained when distance between observations increases, and the semivariogram will be essentially flat. If spatial dependence is present, semivariance will be lower at short distances, it will increase

for intermediate distances, and it will reach an asymptote for long distances.

To adjust data for spatial heterogeneity, we used the iterative spatial analysis (ISA) procedure, as described in Zas [34]. An exponential theoretical semivariogram was fitted to the experimental semivariogram using the NLIN procedure in SAS [28]. This theoretical semivariogram was used to partition the variation of residuals into spatially autocorrelated variation and random error with the kriging method. The kriging estimates at each tree location were used to correct the original values in relation to the spatial heterogeneity, by subtracting the kriging estimates to the original values. The kriging analysis was performed using the KRIG2D SAS procedure [28]. An iterative procedure is needed because, if spatial heterogeneity is quantitatively important, the estimates of the clone effects from the original values could be strongly biased [34]. The clonal breeding value estimates after adjustment for spatial heterogeneity are suppose to be better predictors of true clone effects, and can be used to obtain new residuals from the original data. A new semivariogram and kriging estimates were obtained from these new residuals. These kriging estimates were then used to correct original values, and a new estimation of clone effects was obtained. This process was repeated iteratively until convergence (stability of clone ranks) of the clonal breeding value estimates. A more detailed description of this procedure can be consulted in Zas [34].

2.4. Statistical analysis

Original values adjusted for the spatial structure were analyzed using the following random model:

$$X_{ik} - K_{ik} = \mu + C_i + B_k + \delta_{ik}$$

where X_{ik} is the value of the original variable measured on the *ith* clone in the *kth* block, K_{ik} is the kriging estimate at the position of that tree, μ is the overall mean, C_i, and B_k are the effects of the *ith* clone ($i = 1, 2, \ldots, 122$ for Areas and, $i = 1, 2, \ldots, 71$ for Sergude), and the *kth* block ($k = 1, 2, \ldots, 7$ for Areas and, $k = 1, 2, \ldots, 10$ for Sergude), respectively, and δ_{ik} is the spatially independent error. All factors were considered random. The same statistical model was also used to analyze uncorrected original values.

Variance components and clonal breeding values (BLUPs) were estimated using the restricted maximum likelihood method of the MIXED procedure in SAS [28]. The broad-sense heritabilities were estimated according to Nanson [22]:

$$h^2_{bsi} = \frac{\sigma^2_C}{\sigma^2_C + \sigma^2_e}$$

for the individual broad-sense heritability, and:

$$h^2_{bsc} = \frac{\sigma^2_C}{\sigma^2_C + \sigma^2_e / nb}$$

for the broad-sense heritability based on clone means,

where σ^2_C is the clonal variance, σ^2_e is the residual variance, and b and n are the number of blocks and the harmonic mean of the number of trees per plot, respectively. Standard error of the broad-sense heritabilities ($se(h^2_{bsi})$ and $se(h^2_{bsc})$) were estimated as in Lynch and Walsh [17].

Estimates of the genetic variation coefficient (CV_G) were calculated as

$$CV_G = 100\frac{\sigma^2_C}{\overline{x}}$$

where $\overline{x}$ is the trait mean.

Genotype by environment ($G \times E$) interaction was studied both, among years and between sites. Clone by year interaction ($C \times Y$) was studied in Areas using the following mixed model:

$$X_{ikl} - K_{ikl} = \mu + C_i + B_k + Y_l + C_iY_l + \delta_{ikl}$$

where X_{ikl} is the value of the response variable measured on the *ith* clone in the *kth* block in the *lth* year, μ is the overall mean, K_{ikl} is the kriging estimate at the position of that tree in year l, B_k and Y_l are the effects of the *kth* block, and the *lth* year (l = 2000, 2002, 2004, and 2005), respectively, C_iY_l is the effect of the interaction between the *ith* clone and the *lth* year, and δ_{jkl} is the spatially independent error. All factors were considered random, except the year effect. The model was analyzed with a repeated measurement analysis of variance using the SAS MIXED procedure [28].

Clone by site interaction was analyzed combining the leaf spot data of year 2005 in Sergude with the Areas data of each year. The following mixed model was used:

$$X_{ijk} - K_{ijk} = \mu + C_i + S_j + B_k(S_j) + C_iS_j + \delta_{ijk}$$

where X_{ijk} is the value of the response variable measured on *ith* clone in the *kth* block of the *jth* site, K_{ijk} is the kriging estimate at the position of that tree, S_j and $B_k(S_j)$ are the effects of the *jth* site (j = Areas and Sergude), and the *kth* block within the *jth* site, respectively, C_iS_j is the effect of the interaction between the *ith* clone and the *jth* site, and δ_{ijk} is the spatially independent error. All factors were considered random, except the site factor. Pearson correlation coefficients between clonal breeding values were also estimated to further analyze the $G \times E$ interactions. They were computed using the SAS Proc CORR procedure [28].

The clone stability among years in leaf spot susceptibility was estimated using the S_{4i} stability parameter [12]:

$$S_{4i} = \left(\frac{1}{n} \sum_j \left(r_{ij} - \frac{1}{n} \sum_j r_{ij} \right)^2 \right)^{\frac{1}{2}}$$

where r_{ij} is the rank of the *ith* clone in the *jth* year.

2.5. Relation to bud burst

The relation between fungal disease and bud burst was analyzed by the Pearson correlation between clonal breeding values. Analyses of variance using individual-tree bud burst as covariate were also performed in order to analyze whether differences in clone susceptibility were just due to differences in phenology or whether there were other causes implicated.

3. RESULTS

3.1. Spatial analysis

Residuals after subtracting clone effects revealed nonrandom spatial structures for the fungus disease for all years and

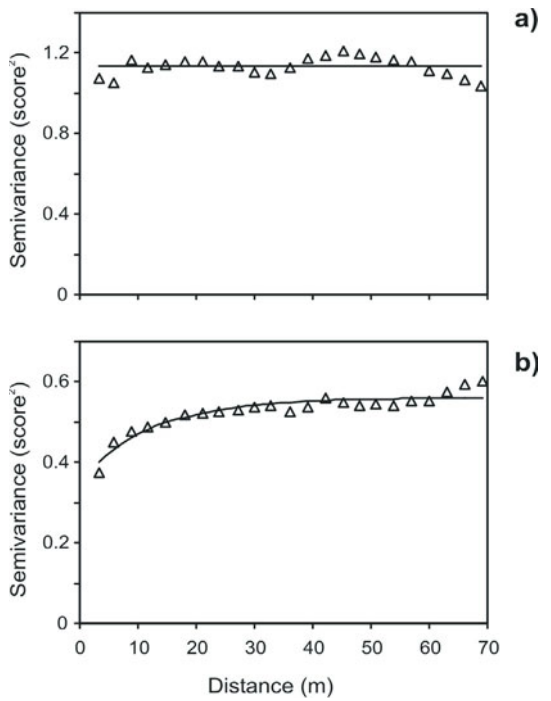

Figure 1. Examples of the semivariograms of residuals after subtracting family effects (fifth iteration) for bud burst (**a**) and cherry leaf spot disease (**b**) in year 2005 in Areas. The flat semivariogram in (**a**) indicated random spatial variation, whereas the reduction of the semivariance at short distances in (**b**) indicated a patchy structure.

sites. The exponential theoretical semivariogram fitted well to the observed semivariogram for cherry leaf spot in all cases ($r^2 > 0.83$, $p < 0.0001$). These semivariograms indicated that data from near neighbors were more similar than those from far neighbors, revealing an spatial autocorrelation. On the other hand, bud burst traits revealed a random spatial structure, as indicated by the flat semivariograms. A comparison between the semivariograms of these two traits for year 2005 in Areas can be observed in Figure 1. The spatial distribution of the residuals for cherry leaf spot susceptibility in year 2005 in Areas is presented, as an example, in Figure 2. The patch size of the exponential theoretical semivariograms for the leaf spot disease varied between 9 and 40 m, and was clearly lower than the block size (~ 0.14 ha in Areas, and ~ 0.2 ha in Sergude), indicating an spatial heterogeneity within blocks that implies a violation of the block design assumptions.

3.2. Clonal variation in leaf spot susceptibility

The variance components, broad-sense heritability estimates, and coefficients of genetic variation for the original values (using the standard analysis) and for the values adjusted for spatial autocorrelation (using the iterative spatial analysis – ISA) are presented in Table I. After adjustment for spatial heterogeneity through iterative kriging differences among blocks disappeared, residual variation clearly diminished, and clonal variation remained constant. The effects on variance components were reflected in a consistent increase of broad-sense heritabilities (13–64% and 4–29% of increase for h^2_{bsi} and h^2_{bsc}, respectively). It should be noted that the spatial analysis procedure also affected the clone ranks which changed up to 30–35 positions after spatial adjustment, although the mean rank change was just six steps (data not presented).

The clonal variance was highly significant in all cases ($p < 0.001$). After spatial adjustment, clonal variance component accounted for 22.4–54.3% and 25.3% of total variance in Areas and Sergude, respectively. Individual broad-sense heritability estimates were moderate to high for all years and sites, ranging between 0.23 and 0.55 in Areas and 0.26 in Sergude. Broad-sense heritability estimates based on clonal means were also high (0.67 to 0.90 in Areas and 0.78 in Sergude). Coefficient of genetic variation ranged between 14.2 and 30.2 in all cases, being almost the double for year 2002 and 2004 in Areas than for the other years and site. Clonal variance and, consequently, broad-sense heritabilities, were clearly lower in Sergude and in the first year of study in Areas, i.e. during the third vegetative period after planting in both cases, which was the first year the disease was observed.

3.3. Interactions between sites and among years

There were 44 common clones at both sites. The joint analyses of variance for both sites (variables adjusted by the ISA procedure) are presented in Table II. Leaf spot susceptibility for year 2005 at Sergude was analyzed together with the Areas data of each year (2000, 2002, 2004 and 2005). Highly significant differences ($p < 0.01$) were found between sites and for the clone by site ($C \times S$) interaction in all cases. Clone variance was also highly significant ($p < 0.01$) for all cases, except when Sergude data was combined with data of year 2000 of Areas. The $\sigma^2_{C\times S}/\sigma^2_C$ ratio diminished as the data assessment year approached, indicating a decrease of the relative importance of the C × S interaction, which was also revealed by the increase of the correlation coefficients (from 0.14 to 0.60, Tab. III) between clonal breeding values as the data assessment approached. Broad-sense heritability estimate for the across-site analyses varied between 0.18 and 0.27 and between 0.61 and 0.75 when based on individual values and on clone means, respectively. Coefficient of genetic variation oscillated between 6.9 and 16.8%.

The repeated measurement analysis for the Areas data showed highly significant differences ($p < 0.001$) among years, clones and for their interaction (Tab. IV). However, the relatively low interaction to clonal variance ratio ($\sigma^2_{C\times Y}/\sigma^2_C < 0.55$) indicated that the $C \times Y$ interaction, although significant, was not high. Moreover, the Pearson correlations (r_P) between breeding values estimated at different years were always significant and higher than 0.47 (Tab. III). Broad-sense heritabilities for Areas, using the repeated measurement analysis, remained high ($h^2_{bsi} = 0.27$ and $h^2_{bsc} = 0.83$) and the coefficient of genetic variation had a value of 15.9%.

Figure 3 shows a biplot between the clone rank stability (S_{4i}) across years and the overall clonal breeding values in

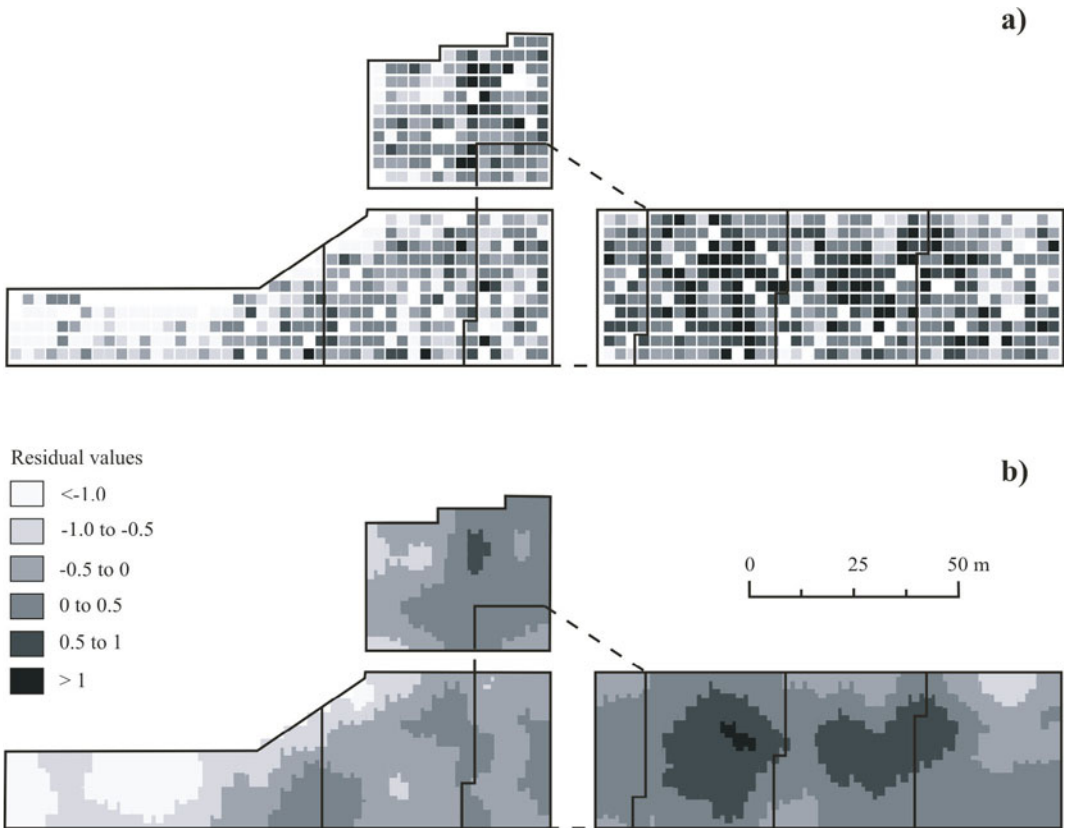

Figure 2. Plot of cherry leaf spot disease residuals for year 2005 in Areas after subtracting family effects showing nonrandom spatial variation (**a**) and modelization of this variation through iterative kriging (**b**). Black lines are the block boundaries.

Table I. Mean values and genetic parameter estimates for cherry leaf spot susceptibility using the standard approach and the iterative spatial analysis procedure (ISA). Standard errors (s.e.) are presented within brackets.

Site	Year	Mean	Standard approach						Values adjusted for spatial correlation (ISA)					
			Clone	Block	Error	h^2_{bsi}	h^2_{bsc}	% CV_G	Clone	Block	error	h^2_{bsi}	h^2_{bsc}	% CV_G
Areas														
	2000	2.94	0.16***	0.02	1.01	0.14	0.52	13.5	0.17***	0.00	0.59	0.23	0.67	14.2
		(1.11)				(0.09)	(0.07)					(0.09)	(0.05)	
	2002	2.08	0.25***	0.01	0.55	0.31	0.76	23.9	0.25***	0.00	0.28	0.47	0.86	24.3
		(0.90)				(0.08)	(0.04)					(0.07)	(0.02)	
	2004	1.66	0.24***	0.00	0.35	0.41	0.83	29.7	0.25***	0.00	0.21	0.55	0.90	30.2
		(0.77)				(0.08)	(0.03)					(0.06)	(0.02)	
	2005	3.56	0.31***	0.01	0.73	0.30	0.75	15.7	0.32***	0.00	0.33	0.49	0.87	16.0
		(1.05)				(0.08)	(0.04)					(0.07)	(0.02)	
Sergude														
	2005	0.76	0.21***	0.11*	0.71	0.23	0.75	17.9	0.19***	0.00	0.56	0.26	0.78	17.4
		(0.48)				(0.11)	(0.05)					(0.11)	(0.05)	

Significance levels: *** $P < 0.001$;** $P < 0.01$; * $P < 0.05$.
h^2_{bsi} and h^2_{bsc}, individual and clonal broad-sense heritability; CV_G, genetic variation coefficient.

Table II. Variance components and broad-sense heritability estimates (h^2_{bsi} and h^2_{bsc}) for the joint analysis of both sites (standard errors within brackets). Cherry leaf spot disease in year 2005 (C05) in Sergude was combined with the different years data in Areas. Variables were adjusted by the ISA procedure (see Material and methods).

	Fixed factors (site)		Variances components				Genetic parameters		
Joint traits Sergude-Areas	$F_{1,15}$	$P < F$	Clone	Block (site)	Clone × Site	Error	h^2_{bsi}	h^2_{bsc}	CV_G (%)
C05-C00	11.8 **		0.04	0.00	0.18 ***	0.58			
C05-C02	32.3 ***		0.13 **	0.00	0.11 **	0.47	0.18	0.61	15.9
							(0.15)	(0.09)	
C05-C04	200.7 ***		0.14 **	0.00	0.07 **	0.42	0.23	0.71	16.8
							(0.14)	(0.08)	
C05-C05	126.1 ***		0.21 ***	0.00	0.08 **	0.50	0.27	0.75	6.9
							(0.14)	(0.07)	

C00, C02, C04 and C05 are cherry leaf spot disease in years 2000, 2002, 2004 and 2005, respectively. Significance levels: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

Table III. Pearson correlation coefficients between breeding values estimated from analysis of variance without bud burst as covariate (above diagonal) and from analysis of variance with bud burst as covariate (bellow diagonal). $N = 44$ for both sites and $N = 122$ for Areas. All coefficients were significant ($p < 0.05$), except between year 2000 for Areas and year 2005 for Sergude.

		Areas			Sergude
Site	Year	2002	2004	2005	2005
Areas	2000	0.56	0.47	0.44	0.14
	2002		0.57	0.53	0.43
	2004			0.64	0.55
	2005		0.44		0.60
Sergude	2005		0.32	0.53	

Table IV. Variance components, broad-sense heritability estimates (h^2_{bsi} and h^2_{bsc}) and genetic variation coefficient (CV_G) obtained from the repeated measurement analysis for Areas data. Standard errors (s.e.) are presented within brackets. Variables were adjusted by the ISA procedure (see Material and methods).

	Random factors		Fixed factors		
Source	Variance components		Source	$F_{3,355}$	$P < F$
Clone	0.165	***	Year	636.6	< 0.0001
Block	0.000				
Clone × Year	0.088	***			
Error	0.349				
h^2_{bsi}	0.27	(.08)			
h^2_{bsc}	0.83	(.03)			
% CV_G	15.9				

Significance levels: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

Areas. Considering that selections should preferably be made for stability and overall good resistance, this figure is very useful for selection purposes. Clones which are at the right-down corner of the graph are those stables, but more susceptible to leaf spot than the mean. On the contrary, clones in the left-down corner (represented inside the dashed ellipsis) are stable and more resistant than the mean.

3.4. Relation with bud burst

The relationship between the clonal breeding values for leaf spot and the clonal breeding values for bud burst at each site is presented in Figure 4. A significant positive relationship between bud burst and leaf spot susceptibility was found in all cases, although it was low for year 2005 in Areas. This means that the earlier the bud burst, the higher the leaf spot susceptibility was. This relationship is also apparent when reanalyzing leaf spot susceptibility data using bud burst as covariate, since the covariate was highly significant ($p < 0.001$) in all cases (Tab. V). Following Singer [29], and Raudenbush and Bryk explanations [24], comparison of clonal variances with and without the covariate indicates that 17.7–65.0% in Areas and 55.5% in Sergude of the explainable variation in clone mean leaf spot susceptibility is explained by mean clonal bud burst (data not presented). Even though, highly significant differences were still found among clones and individual broad-sense heritabilities remained high in this reanalysis. The Pearson correlations between the clonal breeding values for leaf spot susceptibility obtained from the reanalysis were also still high (Tab. III, bellow diagonal).

4. DISCUSSION

The spatial analysis reflected the patchy structure of the *B. jaapii* incidence. The probability of infection was not uniformly distributed in the study areas, and the block designs were not enough to account for this spatial heterogeneity. The conventional statistical analysis may, thus, result in erroneous variance and clonal effect estimates (Tab. I). The iterative spatial analysis procedure used here [34] effectively corrected

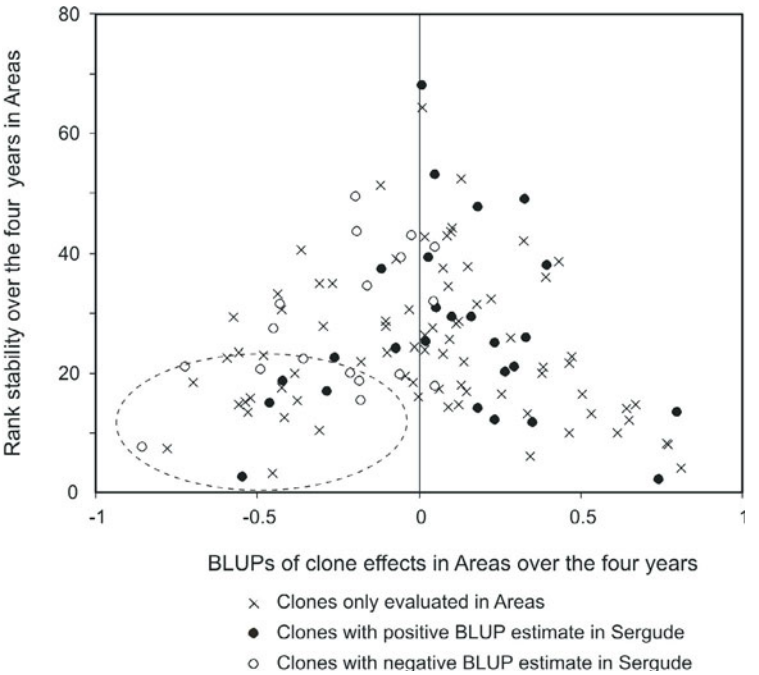

Figure 3. Biplot between overall clonal breeding values and rank stability over the four years in Areas. Clones included in the dashed ellipsis are those more resistant to the fungus disease and stable over time in Areas. Among them, those represented by white circles showed good resistance in Sergude as well.

the data for this spatial heterogeneity. As observed previously in other forest genetic trials [9], heritability substantially increased after adjustment for spatial autocorrelation. This bias in heritability estimates joint to the important changes in the ranking of clones after spatial adjustment widely justified the need of an spatial procedure such as the one used in the present study. The need of spatial adjustments for analyzing spatially correlated data has been emphasized before [6, 8, 9, 34] and could become essential for screening forest tree species for resistance to pathologies as pest incidence is often spatially dependent. As other authors have done before [9,34] we strongly recommend the use of geostatistics to remove spatial autocorrelation in forest genetic trials, especially for screening trees for disease resistance.

After spatial adjustments, the results indicated a high clonal variation in cherry leaf spot susceptibility that was relatively consistent among years and between sites, the fungus infecting some clones significantly more than others. This genetic variation was relatively lower in the first year of study at both sites probably due to the interferences generated by the plantation stress and the lower severity and the higher heterogeneity of the disease incidence during this first year of infection. Other authors have also found a lower genetic control of leaf spot susceptibility on sites with lower attack levels where the scoring of the disease incidence becomes harder [20,26]. Individual broad-sense heritability estimates found in the present work were similar to those obtained by Curnel et al. [2] in several clonal tests of wild cherry in Belgium (h^2_{bsi} = 0.31–0.79), but relatively lower than those reported by Muranty et al. [20] and Santi et al. [26] in France (h^2_{bsi} = 0.77–0.91). The broad-sense heritability estimates on a clone mean basis were also similar to those obtained by Curnel et al. [2].

Despite the $G \times E$ interaction was significant both among years and between sites, it was quantitatively low, and heritability, coefficient of genetic variation and correlations remained high when estimated across years and between sites. These results suggest that the studied clones inherently differed in their susceptibility to the disease but, in some extent, the environmental conditions modulated the expression of this variation. The environmental variation among years and/or sites (namely weather and water and nutrient availabilities) affects both the disease expansion and severity and the plant physiology, resulting in complex interactions in the host-pathogen system. Changes in resources allocation, tissue quality, ontogeny, phenology, and constitutive and/or induced defenses through environmental modulation may be involved in these interactions [5, 7, 16]. In other wild cherry genetic trials results regarding $G \times E$ interaction for leaf spot susceptibility were contradictory. Whereas clone by site interaction was highly significant over 12 clonal tests in Belgium [2], no significant interaction was found over three test sites in France where the high family and clonal stability for leaf spot susceptibility was remarked [20]. In agree with Curnel et al. [2] results, the low interaction to clonal variance ratios and the low decrease in the heritability estimates in the between sites and across years analyses found here suggest little harm in selections due to interactions. Anyway, the significant $G \times E$ interaction implicates that selections for resistance should consider the genotypic stability among years and between sites. Selections should be made for overall resistance and high stability. Thus, the biplot represented in Figure 3 appeared as a useful tool for screening the *P. avium* population for resistance to *B. jaapii*. In particular, those clones represented as white circles (negative breeding values in Sergude) situated in the left-down quadrant of the graph, which have at the same time the lower coefficients S_{4i} (higher stability) and the better resistance at both sites, are the ones that would be the most interesting for breeding selection purposes.

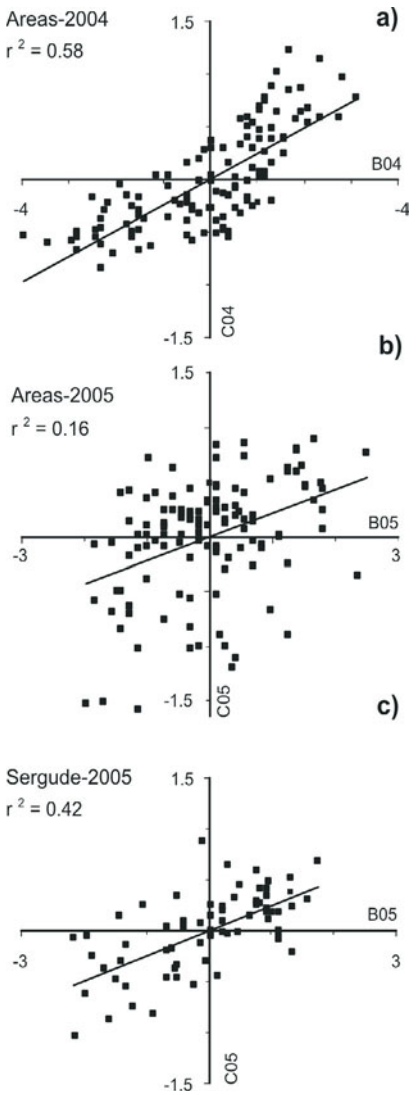

Figure 4. Relationship between clonal breeding values for bud burst and cherry leaf spot disease, for years 2004 (**a**) and 2005 (**b**) in Areas, and year 2005 in Sergude (**c**). Regressions were highly significant ($p < 0.001$) in all cases.

The broad-sense heritability estimates found in the present study are of high relevance for the Galician *P. avium* breeding program. The high values of the broad-sense heritability on a clone mean basis (h^2_{bsc} = 0.67–0.90) joint to the quite high genetic variation (CV_G between 14.2 and 30.2) suggested important genetic gains through clonal selection. In particular working with 2005 data, we would have a genetic gain of 12.5 and 13.1% in Areas and Sergude, respectively, when selecting the 50% most resistant clones, and around 20% in both sites when selecting the 25% most resistant clones (data not presented). Resistant clones can be easily propagated by cuttings or in vitro culture. However, the observed genetic variation in susceptibility (assessed on grafted clones) should be confirmed in cutting or in vitro clonal trials, as the clone ranking for the leaf spot susceptibility may vary in relation to the propagation method [30]. On the other hand, the genetic variation in leaf spot susceptibility may be also exploited by sexual reproduction in the seed orchards, provided that this variation is heritable. The clonal material of the seed orchards should be further analyze by progeny testing in order to provide a further insight in the genetic inheritance of the disease resistance. If family and individual narrow-sense heritabilities are high, the clonal seed orchards should be rouged and highly resistant genotypes could be developed through recurrent breeding. High values of narrow-sense heritabilities can be expected as the additive component of the genetic variance for leaf spot susceptibility has been shown to be the major component (78–89%, [20]). In fact, these authors reported high narrow-sense heritability (h^2_{ns} = 0.37–0.67) for leaf spot susceptibility that allows to launch a recurrent breeding program to develop highly resistant genotypes. Furthermore, the very high correlation between clonal values and general combing ability (0.93) found by Muranty et al. [20] suggested that rouging the clonal seed orchards and selecting the clones to be recombined to produce the next generation population can be made on the basis of the clone effect estimations reported in the present paper.

The determination of the resistance mechanisms would be helpful before starting a breeding program for leaf spot resistance. The results of the present work show that bud burst plays an important role with early flushing clones being significantly more attacked than late ones. As far as we know, this is the first report of such relation, although it was suspected, since the infection of this disease occurs through stomata in early spring. Bud burst explained some of the variation in leaf spot incidence (17–65% of the leaf spot clonal variation is due to mean clonal bud burst), but differences in the disease susceptibility among clones remained highly significant when using bud burst as a covariate in the analyses, suggesting that other genetically controlled factors must be involved in this genetic resistance. The size and morphology of stomata, the foliar tissue chemistry, the presence of repellents, deterrents or toxic compounds, etc. could be implicated. For instance, different glycosides with antifungal activity have been shown to contribute to the defenses to pathogens in different cherry species [13,18], whereas several induced defenses that prevent the proliferation of the fungus throughout the leaf tissue after infection have been also reported in resistant cherry cultivars [33].

Another important question is how breeding for leaf spot resistance will affect other important traits. The results presented here indicated an indirect response in bud burst that would favor late flushing clones. Growth could also be affected as susceptibility to insects and fungus is commonly higher in fast growing genotypes (e.g. [4, 35]). However, this is not likely to be the case in cherry leaf spot, as tree vigor and susceptibility to the disease seem to be negatively correlated [20, 26]. However, this negative correlation may be a cause-effect relation rather than a real genetic correlation, as the disease reduces photosynthesis efficiency and thus growth [23]. The real genetic association between vigor and susceptibility should be thus further analyze using uninfected plants for determining the real clone effects for growth.

Table V. Variance components and broad-sense heritability estimates (h^2_{bsi} and h^2_{bsc}) obtained from the mixed models using bud burst as covariate. Standard errors (s.e.) are presented within brackets. Variables were adjusted by the ISA procedure (see Material and methods).

		Covariate (Bud burst)			Variance components			Parameters		
Site	Year	DF	F value	$P < F$	Clone	Block	Error	h^2_{bsi}	h^2_{bsc}	$\%CV_G$
Areas	2004	1. 712	151.5	< 0.001	0.13 ***	0.00	0.19	0.39	0.82	7.6
								(0.08)	(0.03)	
Areas	2005	1. 591	18.5	< 0.001	0.30 ***	0.00	0.30	0.50	0.87	8.5
								(0.07)	(0.02)	
Sergude	2005	1. 600	86.2	< 0.001	0.09 ***	0.00	0.52	0.15	0.64	12.4
								(0.09)	(0.05)	

Degrees of freedom (DF) for Areas in year 2005 were lower than in year 2004 because one out of the ten replicates was not measured in year 2005.

5. CONCLUSIONS

The *B. jaapii* incidence showed a patchy structure that affected the heritability estimates and even the relative ranking among genetic entries. The iterative spatial analysis based on geostatistics [34] effectively removes this spatial heterogeneity. Breeders and pathologists should consider the use of this method in order to properly screening trees for disease resistance.

There was considerable genetic variation in cherry leaf spot among the studied *P. avium* clones that was relatively consistent among years and between sites. The moderate to high broad-sense heritability estimates suggests that it is possible to include the resistance to the fungus as a selection trait in the Galician breeding program. Important genetic gains can be expected not only through clonal selection but also through sexual reproduction and recurrent breeding.

Cherry leaf spot was related to bud burst, early clones being more attacked than late ones. However, genetic differences among clones for leaf spot were not only due to bud burst, and other unknown genetically controlled factors must be involved in this genetic resistance.

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