Variography and kriging allow screening *Pinus pinaster* resistant to *Armillaria ostoyae* in field conditions

RAFAEL ZAS[1]*, ALEJANDRO SOLLA[2] AND LUIS SAMPEDRO[1]

[1] Centro de Investigacións Ambientais de Lourizán, Apdo. 127, Pontevedra, 36080 Spain
[2] Departamento de Biología y Producción de los Vegetales, Ingeniería Técnica Forestal, Universidad de Extremadura, Avenida Virgen del Puerto 2, Plasencia, 10600 Spain
*Corresponding author. E-mail: rzas.cifal@siam-cma.org

Summary

Armillaria ostoyae is an important disease of *Pinus pinaster* in north-west Spain, which kills trees following a heterogeneous spatial structure. In a progeny trial of *P. pinaster* seedlings, spatial heterogeneity and autocorrelation of neighbour mortality caused by *A. ostoyae* impeded proper analysis of the disease incidence. We used variography and kriging methods to describe the spatial distribution of the infection probability and the genetic variation of the resistance to *A. ostoyae* among families. The spatial structure of disease incidence was modelled, and the probability of survival was corrected by kriging at each tree location. Cumulative mortality 3 years after planting was 65.1 per cent. Significant differences among *P. pinaster* families in terms of mortality to *A. ostoyae* were found, with low individual (h_i^2 = 0.08) and moderate family (h_f^2 = 0.35) heritability estimates. According to a theoretical semivariogram, the patch size of the disease incidence was ~63 m wide. This is the first time variography and kriging are used to select *P. pinaster* resistant to *Armillaria* root rot. It is concluded that geostatistics provides forest pathologists with a powerful tool for screening resistant trees in field conditions.

Introduction

Armillaria spp. cause root disease in natural and artificial forests worldwide, leading to high mortality and important yield losses (Hood *et al.*, 1991; Kile *et al.*, 1991). In conifers of the northern hemisphere, *Armillaria ostoyae* (Romagn.) Herink is the predominant species, being considered as one of the most important pathogens of maritime pine (*Pinus pinaster* Ait.) (Lung-Escarmant and Guyon, 2004). Timber losses caused by *A. ostoyae* in Galicia (north-west Spain), where *P. pinaster* occupies nearly 400 000 ha (Xunta-de-Galicia, 2001), are considerably high.

Armillaria root rot spreads either by rhizomorphs or by root contacts from inoculum sources such as stumps and organic debris where the fungus persists for many years as a saprophyte (Klein-Gebbinck *et al.*, 1991; Redfern and Filip, 1991). *Armillaria* root rot mortality commonly appears in aggregates and the size and shape of these areas vary (Klein-Gebbinck *et al.*, 1991; Van-der-Kamp, 1995; Bruhn *et al.*, 1996; Lung-Escarmant and Guyon, 2004). The infection rate

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depends on the proximity to inoculum sources, and can be expected to follow a spatially heterogeneous pattern (Solla *et al.*, 2000; Lung-Escarmant and Guyon, 2004). Thus, trees living in an *Armillaria* root rot infested area would not have the same probability of infection than trees living in *Armillaria* free areas (Van-der-Kamp, 1995), and screening for resistance to *Armillaria* root rot among different genetic entries in the field would be compromised by the patchy distribution of the probability of infection (Bruhn *et al.*, 1996).

Spatial heterogeneity does not only affect soil pathogens, but it is also a common phenomenon in forest genetic trials evaluating traditional breeding traits such as height or diameter (Fu *et al.*, 1999; Joyce *et al.*, 2002). When spatial heterogeneity is present, near neighbours are more similar than far neighbours, i.e., data are autocorrelated, and the requirement of data independence in standard parametric statistics is violated. This affects both the estimation of genetic parameters and the comparisons among genetic entries (Magnussen, 1993; Fu *et al.*, 1999; Costa-Silva *et al.*, 2001; Dutkowski *et al.*, 2002, 2006; Hamann *et al.*, 2002; Joyce *et al.*, 2002; Zas, 2006). Numerous methods have been proposed to account for this spatial variation, and among them, geostatistics has shown promising results both in agricultural and forest experiments (Fu *et al.*, 1999; Hamann *et al.*, 2002; Zas, 2006). Geostatistics consists of two steps: variography and kriging (Cressie, 1993). Variography uses variograms to model the variation between plots or between individual trees as a function of the distance separating them. When spatial dependence is present, the similarity between plots or between individual trees will decrease as the distance between plots or trees increases. The variogram model obtained is used for interpolation by the kriging method, which provides optimal, smooth and unbiased varying surfaces of values on a spatial grid (Cressie, 1993). The kriging estimates can then be used to adjust the original data for spatial autocorrelation (Hamann *et al.*, 2002; Zas, 2006).

In May 2001, within the frame of the conifer breeding programme undertaken at Centro de Investigacións Ambientais de Lourizán (Galicia), a progeny trial of *P. pinaster* seedlings was established. Three years later, the plantation resulted in an intensively infected *A. ostoyae* area, ideal for analysing the spatial distribution and variable incidence of the disease. Knowledge about the genetic variation within a breeding population such as that for *P. pinaster* in Galicia (see Zas *et al.*, 2004) would be highly desirable because (1) the ability to select trees showing greater resistance would be of enormous financial benefit to the forest industry, and (2) it would provide us with plants with different susceptibilities in which the morphological and physiological mechanisms involved in the resistance could be studied. A first objective of the present work was to use the variography and kriging methods to describe the spatial distribution of the infection probability and the genetic variation of resistance to *A. ostoyae* among open-pollinated families of *P. pinaster*. A second objective was to select resistant families using variography and kriging, which implicates a novel method for screening for disease resistance in field conditions.

Material and methods

Study site and plant material

The study site is located in Laracha, Galicia (north-west Spain, 43° 12' N, 8° 32' W, 250 m a.s.l.). It has an acidic and sandy forest soil above granite bedrock. The soil has a pH of ~4.5, organic matter levels of 17 per cent and low levels of nutrients, especially of phosphorus (Bray-II P = 7 mg kg^{-1}). The climate is maritime, with mean annual precipitation and temperature of 1517 mm and 12.8°C, respectively.

The plant material consisted of *P. pinaster* open-pollinated families obtained from 111 plus trees. The plus trees were selected within the Atlantic-Coast provenance (Alía *et al.*, 1996) for superior growth, stem form and branch characteristics, clonally propagated and their ramets planted in a seed orchard (Sergude, 42° 49' N, 8° 27' W) to provide high genetic quality seeds for reforestation in the Atlantic coast of Galicia. The 111 families comprised almost the whole F_1 breeding population of *P. pinaster* in Galicia. Six unimproved seed lots were used as controls.

The progeny trial was established on May 2001. Site preparation involved the trituration of the dense cover of mainly *Ulex europaeus*,

followed by a 3-m ripening and a superficial scarification. One-year-old containerized seedlings were planted following a randomized complete block design with 25 blocks and one-tree plots. Spacing was 3 × 3 m, and the experimental site occupied an area of ~3 ha. During July 2002, the experimental area was ploughed for weed removal.

Sampling and species identification

In February 2004, when trees were 3 years old, mortality due to *Armillaria* root rot was recorded. Initial inoculum was not possible to quantify following the conventional methods (Onsando *et al.*, 1997; Lung-Escarmant and Guyon, 2004), since no stumps have been left after site preparation. The identification of the pathogen was carried out at Estación Fitopatolóxica do Areeiro, Pontevedra, Spain. Root samples were collected from weak or dead trees at the time of assessments at 10 different locations in the experimental area. Isolations were undertaken on malt extract agar (AM) or benomyl–dichloran–streptomycin media and maintained at 24 ± 1°C in darkness. Species identification of isolates was first carried out by the compatibility method (Korhonen, 1978). Each diploid isolate was compared with haploid reference strains of the six European species: *Armillaria borealis*, *Armillaria cepistipes*, *A. ostoyae*, *Armillaria gallica*, *Armillaria mellea* and *Armillaria tabescens*. Finally, species identification was confirmed by restriction fragment length polymorphism polymerase chain reaction technique (Aguin-Casal *et al.*, 2004).

Spatial analysis

Only trees killed by *Armillaria* were used for further analysis. Residuals of tree survival were used to explore the spatial heterogeneity of *Armillaria* root rot mortality within the experimental area. Mean family survival was calculated and subtracted from each individual observation of the family member. The spatial structure of the resulting residuals was analysed using a semivariogram, which plots the semivariance between individual trees as a function of the distance separating them. The semivariance $\gamma(h)$ was calculated as

$$\gamma(h) = \frac{1}{2n} \sum_{i=1}^{n} [z(s_i) - z(s_{i+h})]^2 ,$$

where n is the number of observation pairs separated by distance h (called the lag distance), $z(s_i)$ is the value for a tree located at s_i and $z(s_{i+h})$ is the value for a tree located at a distance h from s_i. For randomly distributed data, little change in the semivariance will be obtained when h 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 typically reach an asymptote for long distances. The distance at which the asymptote begins, if present, indicates the range or patch size of heterogeneity below which data are stochastically dependent (Cressie, 1993). By common convention, the analysis is restricted to distances of half the dimension of the study area. The experimental semivariogram was constructed using the VARIOGRAM procedure of the SAS System (SAS-Institute, 1999).

Spherical and exponential models were fitted to the experimental semivariogram using the NLIN procedure in SAS (SAS-Institute, 1999). Since the exponential model fitted better than spherical model, this model was used to partition the variation of survival residuals into spatially autocorrelated variation and random error with the kriging method. Kriging computes surfaces of best linear unbiased predictions of values based on the spatial structure defined by the theoretical semivariogram. In our case, this surface was interpreted as the spatial distribution of the probability of survival of trees to *Armillaria* root rot. Thus, the kriging values at each tree location were used to correct the original survival values in relation to the spatial variation. Because being alive in a free *Armillaria* zone is not the same as being alive in a high-risk infection zone, original survival values were corrected by removing the kriging estimate. This new variable varies, theoretically, between –1 (dead plant in a totally *Armillaria* free area) and +1 (non-symptomatic plant in a completely infected area) and was considered as an indication of the resistance to the disease. The kriging analysis was performed using the KRIGE2D SAS procedure (SAS-Institute, 1999).

Genetic parameter estimates

Genetic variation in resistance to *Armillaria* root rot disease was analysed by the following random model:

$$S_{ij} = \mu + F_i + B_j + \epsilon_{ij},$$

where S_{ij} is the value of the corrected survival of family i in block j, μ is the overall mean, F_i and B_j are the random effects of family i (i = 1–111) and block j (j = 1–25), respectively, and ϵ_{ij} is the random error. Variance components of random effects were estimated using the restricted maximum likelihood (REML) method of the MIXED procedure in SAS (SAS-Institute, 1999). Individual and family heritabilities were calculated upon the variance components as described in Falconer and Mackay (2001). Standard errors were estimated using the formulae described in Wright (1976).

Results

Overall mortality 3 years after planting was 65.1 per cent. Most trees died during summer and autumn 2003. Within the F_1 population, family mortality rate varied between 40 and 88 per cent. Control seed lots mortality rate varied between 56 and 76 per cent. More than 95 per cent of the dead trees presented typical mycelial fans under the bark and ~90 per cent of recently killed trees presented white mycelium at the root collar. Both the compatibility method and the RFLP-PCR detected *A. ostoyae* in root samples.

The spatial heterogeneity of the incidence of *Armillaria* root rot disease was evident from the plot of survival residuals (Figure 1a), some areas being much more affected than others. This heterogeneity was confirmed by the experimental semivariogram (Figure 2) which clearly indicated that neighbouring trees tend to have more similar values than trees far away.

The exponential function fitted well to the experimental semivariogram (Figure 2), with a regression coefficient between observed and expected values of 0.94 ($P < 0.001$), and other typical functions such as the spherical or Gaussian models provided less significance. The resulting theoretical semivariogram was $\gamma(h) = 0.1918 + 0.0273\ (1 - e^{-h/21.0405})$, where h is the distance between trees (m). In exponential semivariograms, three times the value $a_0 = 21.0405$ is often used as the effective range indicating the distance in which values are spatially dependent (Webster and Oliver, 1990). In consequence, the patch size of the *Armillaria* incidence was about 63 m (3×21.0405 m). The obtained semivariogram was used to model the spatial variation with kriging (Figure 1b).

The analysis of the corrected survival (survival minus kriging estimate) revealed significant

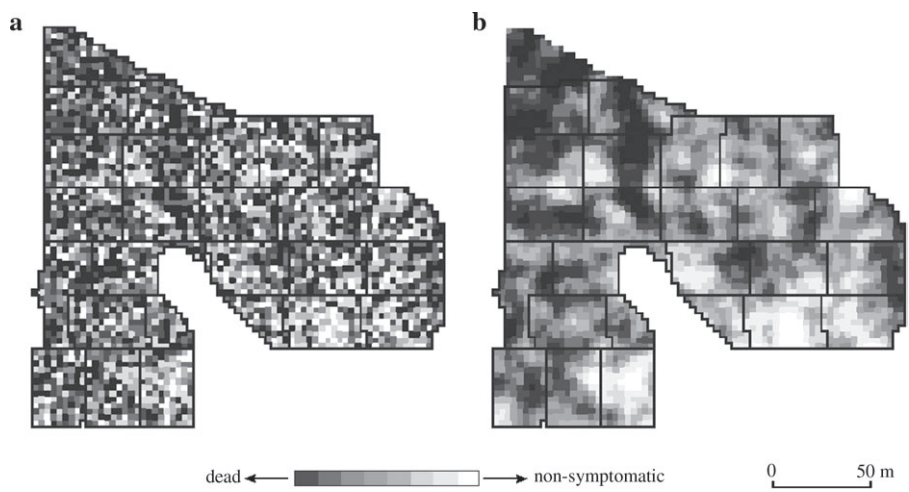

Figure 1. Spatial distribution of survival of *Pinus pinaster* seedlings to *Armillaria ostoyae* adjusted for family effects (mean family survival subtracted to each observation) (a), and spatial model of survival estimated by the kriging method (b). The map in the right can be interpreted as the probability of trees being killed by *A. ostoyae*. Each pixel is a tree unit. Bold lines are the block boundaries. Dark shades indicate dead and light shades represent non-symptomatic plants.

differences among families as indicated by the family variance significantly greater than zero (Table 1). Since all environmental variations were removed when correcting the original survival values, the block effect was null. Individual heritability estimate was low (h_i^2 = 0.08 ± 0.03) and family heritability was only moderate (h_f^2 = 0.35 ± 0.04). According to the model, families labelled as 1000, 2036, 1012, 2037, 2024 and 2026 were those that showed the highest survival rates to *A. ostoyae*.

Discussion

The techniques used in this study allowed not only to describe the spatial distribution of *Armillaria* root rot incidence within an experimental area but also to identify resistant *P. pinaster* families taking into account the spatially heterogeneous nature of the disease. The spatial analysis reflected the patchy structure of the *A. ostoyae* incidence. Previous epidemiological studies have shown similar results although the extent of aggregates varied substantially. Lung-Escarmant and Guyon (2004) found *A. ostoyae* inoculum to be heterogeneous spatially distributed in a *P. pinaster* plantation, with a variogram range value of ~21 m. In a Douglas-fir (*Pseudotsuga menziesii*) stand, Van-der-Kamp (1995) found *A. ostoyae* to occur in smaller domains of just a few metres in diameter, whereas (Klein-Gebbinck *et al.*, 1991) did not observe any spatial association between dead or dying trees. On the other hand, the spatial pattern of *Armillaria* root disease mortality in *Picea mariana* seed orchards was found to be aggregated on a scale larger than 0.2 ha (Bruhn *et al.*, 1996), similar to our findings. The estimation of the spread rate of *Armillaria* root disease has also been shown to vary widely (Van-der-Kamp, 1993; Peet *et al.*, 1996) indicating the variable nature of the disease in different sites. As pointed out by Van-der-Kamp (1993), this calls attention to the need to use site-specific measures.

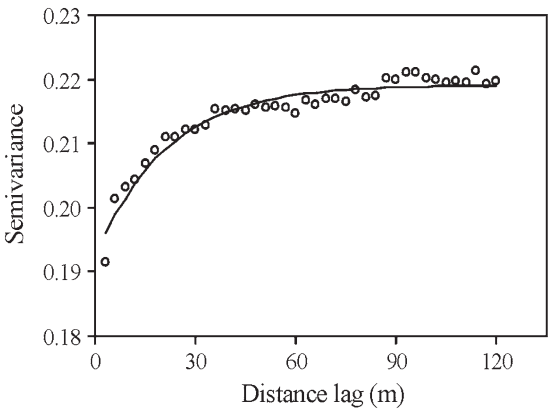

Figure 2. Semivariogram for survival of *Pinus pinaster* adjusted for family effects, i.e. survival family mean subtracted to each individual observation.

Geostatistics was also successfully used to remove spatially autocorrelated variation of growth traits in *Alnus rubra* and *P. pinaster* genetic trials (Hamann *et al.*, 2002; Zas, 2006). In these works, family and provenance variance components increased up to 40 per cent in some traits at the spend of block and family × block interaction effects, heritability increasing accordingly. Previous authors concluded that (1) variograms should be routinely inspected to analyse forestry field trials, and (2) the kriging method should be used for adjustments when spatial heterogeneity is detected. Kriging was also successfully used to model the density and distribution of *Armillaria* inoculum in a *P. pinaster* stand in Landes de Gascogne (southwest France) (Lung-Escarmant and Guyon, 2004). Other spatial analyses have proved useful for surveying the spread of plant pathogens and the

Table 1: Variance component estimates, significance levels and individual and family heritability estimates for the corrected survival, i.e. survival adjusted for spatially autocorrelated variation

Effect	Variance estimate	*Z*-value	$P < Z$	h_i^2	h_f^2
Family	0.0032 + 0.0012	2.58	0.0050	0.08 + 0.03	0.35 + 0.04
Block	0.0000 + 0.0000	–	–		
Residual	0.1459 + 0.0040	36.39	0.0000		

incidence and/or severity of tree diseases (Coulston and Riitters, 2003; Solla and Camarero, 2006; Díaz *et al.*, in press). According to the results presented here, geostatistics provides us with a powerful tool for screening for resistance in a naturally and spatially heterogeneous infested area.

The incidence reported here (65 per cent) was much higher than in other *A. ostoyae* infested areas (e.g. Klein-Gebbinck *et al.*, 1991; Smith *et al.*, 1994; Bruhn *et al.*, 1996; Solla *et al.*, 2000; Lung-Escarmant and Guyon, 2004). High and widespread tree mortality was probably caused by the large amount of small woody debris dispersed during site preparation, which leads to an increase of inoculum potential (Smith *et al.*, 1994; Onsando *et al.*, 1997; Solla *et al.*, 2000). Further soil practices such as ploughing for weed removal, had probably scattered the initial inoculum. This inoculum was probably the main cause of disease incidence, and it is expected to play a major role in future tree mortality (Lung-Escarmant and Guyon, 2004).

Genetic variation of resistance of *P. pinaster* to pathogenic fungi and insects has been previously reported. Differences in susceptibility to *Melampsora pinitorqua* were found among *P. pinaster* provenances (Desprez-Loustau and Baradat, 1991), and among families within the French *P. pinaster* breeding population, with a heritability estimate of 0.06 (Baradat and Desprez-Loustau, 1997). Intraspecific variation in *P. pinaster* resistance to different insects such as *Matsococcus feytaudi* (Burban *et al.*, 1999), *Dioryctria sylvestrella* (Kleinhentz *et al.*, 1998) and *Hylobius abietis* (Zas *et al.*, 2005) has also been reported.

Few experimental studies are available to explain the variation in resistance to *Armillaria* root rot found in tree species. Susceptibility to this disease widely varies among tree species (e.g. Omdal *et al.*, 1995; Baumgartner and Rizzo, 2001) and literature suggested a link between this variation and the phenolic/sugar ratio found in the root bark (Entry *et al.*, 1992; Myszewski *et al.*, 2002). Myszewski *et al.* (2002) found differences in bark chemistry among *Pseudotsuga menziesii* families in several progeny trials and suggested that this variation could be exploited through breeding to obtain *Armillaria* resistant genotypes. Differences in host susceptibility among *Picea abies* provenances to *A. ostoyae* have also been reported (Prospero *et al.*, 2004). Considering differences in the formation of lingo-suberized boundary zones among *Picea sitchensis* trees after inoculation with *A. ostoyae*, the possibility of improvement through selection for resistance was indicated (Solla *et al.*, 2002). In accordance with these reports, the present study is, to our knowledge, the first to show genetic variation in terms of resistance to *Armillaria* root rot at field conditions.

From a statistical point of view, our method is probably overestimating the heritability results since the kriging values used to adjust the original values are estimations obtained with a certain degree of uncertainty. This uncertainty would imply a reduction of the residual degrees of freedom in the analysis of the adjusted data, which would probably led to a reduction of the estimated family variance. Statistical packages such as ASReml (Gilmour *et al.*, 1999) or even the MIXED procedure of SAS (see Littell *et al.* 1996, Saenz-Romero *et al.* 2001; Hong *et al.*, 2005) would allow to do the smoothing and estimation within one analysis, incorporating the spatial structure of the residuals in a single step, with all necessary adjustments allowed for. With this type of analysis, however, valuable information related to the spatial structure of the data would be lost, and the analysis of binary data would be problematic. Adjusting values by subtracting the kriging estimates generates a continuous variable that varied from –1 to 1, which is easy to analyse. Littell *et al.* (1996) warned about the incorporation of residual spatial structures in the MIXED procedure for the analysis of large datasets such as the one presented here. Rather, they recommended to externally fit a semivariogram to the residuals of the model fitted by REML, and incorporate the resulting parameters of the theoretical semivariogram as known spatial covariance values in the MIXED procedure. However, convergence of REML becomes harder as the number of restrictions to the parameter estimations increases, and in our case, REML failed to converge when the spatial covariance parameters obtained from Figure 2 where specified in the MIXED procedure.

From a biological point of view, the possible variation of virulence shown by the isolated *A. ostoyae* strains within the experimental area, if any, would be the strongest constraint of the present work. In addition, the classification of symptomatic trees into a severity scale could have improved the precision of the disease incidence and reduced the problems derived from analysing 0/1 values. A survival time

analysis where resistance is interpreted as delayed mortality (e.g. He and Alfaro, 2000) could also have improved the results if the influence of plant age on symptom development was considered. Nevertheless, results presented here indicated significant differences among *P. pinaster* families in relation to survival to *A. ostoyae*, allowing a first screening of trees. The selected resistant families can be recommended for planting in sites in which *A. ostoyae* has been demonstrated to be a potential problem.

Geostatistics enables the quantification of the spatial distribution of diseases, allowing a more realistic modelling of disease occurrence, impact and risks. We strongly suggest forest pathologists and breeders to use the geostatistic method reported here in order to properly analyse field data typically affected by autocorrelation and spatial heterogeneity, thus allowing to a more reliable selection of resistant trees.

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