Title

Soil spatial distribution in a smut fungus-annual grass interaction: Exploring patterns to understand disease dynamics at plot scale

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Abstract

Infection of large crabgrass by loose smut occurs in the first centimetres of the soil profile. To understand the spatial-temporal disease dynamics, we try to find out the importance of the spatial distribution of spores and seeds in a field. Two soil sampling strategies were carried out after doing soil tillage, and propagules were counted; in the following season the level of disease was assessed. There was spatial autocorrelation in the case of the spores, but not in that of the seeds. Spores were arranged in a single patch. A significant relationship was found between the density of spores and the percentage of diseased host plants in the next season. Both variables displayed a spatial structure based on multiple regression. The role that the different zones of the field, varying in spore abundance, may play in the coexistence of both partners is discussed in the light of the results.

Keywords

Ustilospores, spikelets, plant disease, spatial analysis, within-population variation, *Digitaria sanguinalis*, *Ustilago syntherismae*

Introduction

Soils contain very substantial numbers of phytopathogenic organisms, some of them having very limited and highly specific host ranges while others are generalists (Dixon & Tilston 2010). Among plant pathogens, fungi constitute a very large and heterogeneous group, and show an enormous diversity in life-history strategies and the ways in which they interact with their hosts (Burdon & Silk 1997; Termorshuizen & Jeger, 2008). Together with the comprehension of life histories, spatial structure is central to understanding the dynamics of interactions (Burdon & Thrall 2008), particularly in wild plant-pathogen systems, that commonly exhibit considerable spatial variability at all levels of scales (Burdon 1993).

In the current paper, we present a *Digitaria sanguinalis* (L.) Scop. - *Ustilago syntherismae* (Schwein.) Peck pathosystem in which plant infection, as far as we know, can only occur in the soil. *U. syntherismae* is a smut fungus (Basidiomycota, Ustilaginomycetes, Ustilaginales; Minnis *et al*. 2017). All smut fungi, including Ustilaginomycetes, are plant pathogens ecologically well characterized by their parasitism of vascular plants (Bauer *et al*. 2008). The species under study is biotrophic, that is, the fungi

establish a long-term feeding relationship with living cells of the host plant, growing internally, and cause a disease called loose smut. The only unequivocal morphological disease symptom at macroscopic level is the smutted inflorescence. In this way, the inflorescence is replaced by sori with thick-walled ustilospores, at first hidden by developing leaf sheaths and later more or less visible (Vánky 1994). *U. syntherismae* can cause systemic infections of several species of *Digitaria* Haller nearly worldwide (Minnis *et al*. 2017). Its effects were evaluated by Johnson & Baudoin (1997), who assessed its potential for biological control of *D. ciliaris* (Retz.).

D. sanguinalis is a representative of the family *Poaceae*. It is known as a cosmopolitan annual weed characteristic of summer crops, even glyphosate-tolerant ones (e.g., Puricelli & Tuesca 2005; Dewar 2009; Mas *et al*. 2010), and in temperate regions does not have asexual reproduction mechanisms. The ustilospores formed in mature diseased plants, either individually or enclosed in the sori, reach the soil at the same time as the one-seeded spikelets formed in healthy plants. The future inoculum overwinters in the soil or attached to the spikelets present also in the soil (for instance on the surface of the scales: glumes, lemmas or paleae), and finally the new infection takes place during the very early seedling stage. In some way, as Meyer *et al*. (2016) mention for *Ustilago bullata* interacting with *Bromus tectorum*, the two organisms would follow a coevolved pattern in which dormancy is lost in springtime, grass caryopsides and smut spores germinating in parallel with a certain degree of synchrony. To reduce the *D. sanguinalis* soil seedbank in the summer crops, fields should be subject to a long-term weed management strategy. Because in this species the seed viability decreases to about 70% after almost one year of burial, with practically no seeds surviving after three years (Masin *et al*. 2006), the possible establishment of the interaction with *U. syntherismae* and the prevalence of the disease for several years could contribute to the decrease of the annual seed rain and therefore the viable soil seedbank.

We observed smut heads on the grass plants for the first time in 2004 (Mas *et al*. 2006) in an uncropped edge of an arable field near Barcelona. From then until the present, year after year, the disease has prevailed in that field, even though no alternative host species has been detected in the area and no other diseased large crabgrass population has been observed in the vicinity either. In the population that we monitored, excluding plants that escaped infection because they were not in contact with ustilospores, four levels of within-population variation in host resistance can be distinguished, taking into account the results of the laboratory trials with forced infection (Mas & Verdú 2014): (i) plants that avoid infection; (ii) infected plants that do not develop the disease; (iii) partially tolerant infected plants that develop seeds

and spores; and (iv) fully susceptible plants. Moreover, it is known that the ustilospores formed on partially diseased plants are less infective than those formed on completely smutted plants, giving two levels of within-population variation in infectivity (Jorba *et al.* 2015). The local persistence of the pathosystem or, in other words, the ability to coexist, can only be understood if there are mechanisms that maintain and/or generate both the fungus variation in infectivity and aggressiveness and the plant variation in qualitative and quantitative resistance at population level. The key question is how they coexist.

Laine *et al.* (2011) point out that a key aim for host-pathogen research is to generate a consensus on how life histories, spatial structure and population dynamics all interact simultaneously to determine patterns of genetic and phenotypic variation in virulence and resistance. With respect to coevolutionary dynamics, recent reviews in plant-pathogen interactions (Burdon & Thrall 2009; Laine *et al.* 2011; Tack *et al*. 2012) gather empirical and analytical evidences that resistance and virulence polymorphisms can be maintained in accordance with two models: (i) fluctuating selection dynamics, where host and pathogen genotype frequencies oscillate over time because of negative frequency-dependent selection, and (ii) directional arms race dynamics, where both species continually accumulate adaptive mutations, with escalation of defence and counter-defence. Nevertheless, most of the studies that support these coevolutionary models are based on inoculation experiments in which there is no restriction on the contact between the pathogen and the plant. Moreover, the number of studies on smut-plant interactions is still limited, and most of them focus on pollinator-transmitted smuts. In this way, the same reviews emphasized the lack of empirical studies that could confirm or refute the theoretical ones, and claim that many assumptions of the theoretical models are rarely fulfilled or untested in particular plant pathogen systems. Specifically, both host and pathogen life-history traits (e.g., reproductive system, dispersal mechanisms) can influence the patterns of genetic variation (Barrett *et al*. 2008; Burdon & Thrall 2009). The third element in play in the coevolutionary scenario is spatial structure, which is considered essential by Laine *et al*. (2011) in their review, because spatially heterogeneous selection is demonstrated to be equally important for the maintenance of virulence and resistance variation at multiple spatial scales. In many of the cases that have been studied the spatial structure was linked to environmental variation patterns (e.g., García-Guzman *et al*. 1996; Antonovics 2009; Laine 2007; Penczykowski *et al.* 2015).

Moreover, the whole picture would remain incomplete if we were not to consider disease escape, defined by Burdon (1987) as a simple spatial phenomenon that implies lack of infection resulting from

the failure of host and pathogen to come into physical contact although both are present in the same environment at the same time.

As mentioned before, in *U. syntherismae-D. sanguinalis* interaction, the first condition to be met is for the ustilospore to find the seedling. In terms of probability, infection depends on the location of the host relative to sources of infection (Real & McElhany 1996), which is expected to have a particular spatial arrangement, as soil organisms show spatially predictable and aggregated patterns over scales ranging from hectares to square millimetres (Ettema & Wardle 2002). Verdú & Mas (2015), in a four-year study on the prevalence of this monocyclic disease in the abovementioned field, found that the percentage of diseased plants increased when the plant density increase, which is unexpected in monocyclic diseases. As the authors argued, density dependence should not be explained by the infection process *per se*, but could be related to the density of both fungal inoculum and seeds in the soil, besides the spatial distribution of both propagules. In the conceptual framework that has been set forth, the possibility of disease escape is not necessarily uniform from the spatial point of view, and for this reason it is worth studying this subject, which would help us to understand the persistence of the *U. syntherismae-D. sanguinalis* interaction, and this could be of interest not only from a weed control point of view but also as a contribution to increase knowledge of plant-pathogen coevolutionary dynamics.

The main purpose of the present work is to find out whether there is spatial pattern in the overwintering soil propagules of *U. syntherismae* and *D. sanguinalis* populations, and if so, to describe it and discover how it is related, if at all, to disease incidence in the next generation.

Materials and methods

Study site

The research was performed in 2012 and 2013 on a 15x30 m^2 study plot that forms a corner of a field located near Barcelona, at the Institut de Recerca i Tecnologia Agroalimentàries experimental station (Torre Marimon, Caldes de Montbui, 41°36’44’’N and 2°10’17’’E 1001700E / UTM 31N 4607078N 431060E). The climate in the area is temperate Mediterranean. The mean annual precipitation is 600 mm and the monthly mean air temperature is 14.5°C, ranging from 6.5°C in January to 23.5°C in July. The soil was an Inceptisol sandy loam Calcixerollic Xerochrept (Josa *et al.* 1984) located on an alluvial terrace with carbonated alluvial deposits as parent materials (IGME 1976).

The field was under crop production until 2006, when the study of the *D. sanguinalis-U. syntherismae* interaction was started. From 2001 to 2006, winter barley crops were grown, and before those, two years of sunflower and several years of maize watered by sprinkler were grown, all of them under a conventional mouldboard ploughing tillage system. As of 2007 no crop was sown, but chisel ploughing at a depth of 20 cm was still done in April or May, prior to the first flush of *D. sanguinalis* seedling emergence, and in November, after the plants had been killed by frosts. Since the winter of 2009, when the whole field except the study plot was occupied by perennial crops using black geotextile mulches for weed control, the machinery passes had always followed the same work pattern. This consisted of eight to twelve parallel passes (depending on the width of the farm equipment) along the length of the plot from WNW to ESE, and one or two transverse passes along the width at the two ends of the plot from SSW to NNE (Fig 1).

Under this soil disturbance regime, which was done if a summer crop was sown in the field, the plant community that developed each summer belongs to the phytosociological alliance *Panico-Setarion* (Masalles 2008) and has more than twenty different plant species. Besides *D. sanguinalis* plants, among those with high densities there were populations of *Amaranthus retroflexus* L., *Chenopodium album* L., *Convolvulus arvensis* L., *Cynodon dactylon* (L.) Pers, *Datura stramonium* L., *Euphorbia prostrata* Ait., *Paspalum distichum* L., *Portulaca oleracea* L., *Setaria verticillata* (L.) P. Beauv., *S. viridis* (L.) P. Beauv., and *Xanthium strumarium* L. These plant communities of the abovementioned alliance, mainly composed of typical agrestal species, can be found in Mediterranean farmland characterized by several crops (such as vineyards, olive and almond groves, and winter cereals under dry-land conditions, as well as fruit orchards and maize fields under irrigation). They also include forested areas (particularly secondary pine forests). The whole forms a mosaic landscape with a network of tracks and roads that assume the role of corridors. The species of these plant communities can colonize crop field margins or orchard inter-rows, but they are particularly common in the winter cereal fields that are ploughed after harvesting as a measure to prevent the spread of summer fires (Marull *et al*. 2008) and left unsown until the next winter.

Pathosystem

Since 2006, the *D. sanguinalis-U. syntherismae* pathosystem of this field has been under study, with attention to some aspects concerning demography, the effects of the systemic infection on plant fitness, the phenology and morphology of smutted and non-smutted plants in a range of densities, as well as some

key life-history traits, particularly infection (Gallart *et al*. 2009; Mas & Verdú 2014; Verdú & Mas 2015). Among the four modes of infection that occur in smut species (Kronstad 1996), to our knowledge the ustilospores of *U. syntherismae* penetrate the *D. sanguinalis* host when, after seed germination, the young seedlings are growing to reach the soil surface. No basidiospore was seen to bud from the external basidium in any of the microscopic examinations performed on in vitro germinations of *U. syntherismae*. The germinating hypha is probably the infective hypha, as in other described smuts in which the dicaryotization occurs within the ustilospore or at very early germinating stages of it (Ingold 1989; Piepenbring *et al*. 1998). Once an entry occurs, as happens with biotrophic fungus, hyphae can grow in association with vascular bundles and also extend into the parenchyma. In zones with temperate climates, in which *D. sanguinalis* is a summer annual, the disease is monocyclic, i.e., the *U. syntherismae* ustilospores cannot reinfect plants within the season. According to Jarosz & Davelos (1995), this smut fungus can be considered a specialized soil-borne fungus which has a restricted host range (in our scenario only *D. sanguinalis*). The percentage of self-pollination in *D. sanguinalis* is very high (Lemen 1980) and, as mentioned before, it is possible that the *U. syntherismae* dicaryotization occurs within the ustilospore or the probasidium germinating stage. Thus, the genetic recombination rate might be low for both partners.

Data collection

Soil spikelets and ustilospore sampling

The sampled area within the study plot was 14 m wide and 26 m long. Two sampling strategies were performed to collect data: (i) a systematic method in which a soil core was taken in each of the 112 nodes of a 2 m x 2 m grid, and (ii) another method in which 60 soil cores were taken at random from among the 405 positions of a 1 m x 1 m grid; 23 of these nodes coincided with those of the systematic method, and therefore were sampled only once (Fig 1). The random sampling allowed us to identify and describe the spatial structure of spikelets and ustilospores, while the systematic sampling was used to estimate the spatial parameters to model for prediction purposes, and also to test the relationships between ustilospore and/or spikelet soil densities and degree of disease incidence recorded in the following summer.

The cores were 1.5 cm in diameter and 5 cm long, and were taken from the top 5 cm of the bare soil in December 2012, immediately after one pass with a horizontal axis rotary cultivator followed by a rule pass. The soil was previously chisel ploughed to a depth of 20 cm, in November 2012. Considering the preceding studies on large crabgrass emergence at different depths (Benvenuti *et al*. 2001), only

spikelets buried at less than 5 cm had a chance to germinate and emerge. Moreover, 44.2 mm (SEM 0.54 mm) was the maximum mean mesocotyl length achieved by the seedlings when spikelets harvested in 2011 at the study plot were checked in a laboratory trial simulating field conditions (Mas & Verdú 2016).

The soil core samples collected were taken to the laboratory in plastic bags and stored at -20°C until processed. Each core was defrosted and subjected to Carretero's method to disperse soil particles and remove seeds (Carretero 1977). The *D. sanguinalis* spikelets containing caryopsides trapped in the sieves were counted. The solution containing particles smaller than 300 µm, not retained by the sieves, was centrifuged at 9500 rpm for 10 minutes using a laboratory tabletop fixed-angle centrifuge; the sediment was rejected, and the supernatant was filtered using a Kitasato funnel applying vacuum with Whatman N. 60 filter paper. The filter paper was divided into three portions of equal surface area. The central one was placed between two microscope slides that also enclosed an acetate grid; the mounting medium was water. The filter paper portion was observed scanning the entire surface at x100 using a light transmission microscope and spores that were recognized as *U. syntherismae* were counted. Thus, the raw data were the number of spikelets containing caryopsides of *D. sanguinalis* per soil core (SEEDSc) and the number of spores of *U. syntherismae* per one third of the soil core (SPORES1/3c).

Plant sampling

In 2013, after the disturbance of soil tillage in the spring, the whole plot was sampled with 35 permanent quadrats, each measuring 0.25 m^2, placed at intervals of 3 m along five transects 3 m apart. The precise location of each quadrat within the plot was obtained by measuring the distances from one of the vertices to two reference points, both also used in the soil sampling, using a Leica DISTO™ Plus laser distance meter (Fig 1). After the first flush of *D. sanguinalis* emergence the seedlings of other plant species that appeared within the quadrats were removed weekly, and only *D. sanguinalis* was allowed to grow within them.

At the end of the annual cycle the surviving *D. sanguinalis* plants in each quadrat were collected, counted, and sorted according to their disease status. The proportion of diseased plants was obtained by dividing the sum of the completely and partially smutted plants by the number of plants bearing spores and/or spikelets.

Data analyses

Descriptive statistics related to data distribution, such as quantiles and extreme values, together with frequency histograms, were computed using the SAS/UNIVARIATE procedure (SAS 2013). Considering

together all 149 cores sampled, two outliers were rejected, one of spikelet count and the other of ustilospore count, both belonging to the systematic sampling.

The data collected from the soil samplings, SEEDSc and SPORES1/3c, the latter previously decimal logarithm-transformed ($\log_{10}$SPORES1/3c), were analysed to find out firstly whether both propagules were similarly distributed in the soil, presumably facilitating physical contact between them, or not. After that, count data were analysed in order to obtain contour maps with estimations of propagule densities at unsampled locations of the field, and finally these predictions were used to search for relationships between the estimated propagule densities in the soil during winter and the disease incidence observed the following summer.

Specifically, four types of analysis were performed: (i) The variance-to-mean ratio, frequently used as a dispersal index, to roughly compare between seeds and spores. (ii) Count data were fitted to the Poisson probability distribution and to the negative binomial probability distribution by means of logistic regression using the SAS/GENMOD procedure, and parameters were estimated using a complementary log link function and Type III analysis options; the deviance divided by the degrees of freedom was used as the criterion for assessing the goodness of fits. The use of probability distributions to characterize the spatial patterns of count data is a well-established technique in population and community ecology, including the study of the spatial patterns of soil-borne pathogens (Nicot *et al*. 1984). We compare the goodness of fit of two probability distributions, and if necessary we use the parameters of the functions. (iii) For each metre lag distance between 2 m and 14 m the Moran's *I* coefficients of spatial autocorrelation (Moran, 1950) and its standard deviations were obtained for both SEEDSc and SPORES1/3c under a randomization assumption using the SAS/VARIOGRAM procedure, and therefore spatial correlograms were drawn in order to identify and describe the spatial intensity of any possible spatial pattern (Fortin & Dale 2005). (iv) The same SAS procedure was used to plot, with the data from the systematic sampling, experimental omnidirectional variograms (semivariance with respect to lag distance) for spikelets and for ustilospores, in order to characterize the spatial structure of the data in terms of dissimilarity between observations (Fortin & Dale 2005) and, if possible, to model them as theoretical variograms. Because anisotropic spatial patterns could not be ruled out *a priori* due to possible effects of the machinery passes, four experimental directional variograms were also computed at 0º, 45º, 90º and 135º angles.

A contour map of predicted spore density ($Predlog_{10}SPORES$) was drawn by means of the SAS/GCONTOUR procedure with the information obtained after the following steps: (i) removing the surface trend of $log_{10}SPORES1/3c$ by means of an analysis of covariance performed using the SAS/GLM procedure. Thus, the spore density was analysed by computing Type III sum of squares considering the plot coordinates as covariables (width, length, and their quadratic forms) and the residuals were calculated. (ii) Exploring, once more, whether or not there was anisotropy experimental variograms following four directions were computed with the residuals. (iii) The weighted sum of squares method was used to fit the semivariogram of the residuals to any model using the SAS/SEMIVARIOGRAM procedure. (iv) The best fitted model was used to calculate the estimated values for residuals by means of ordinary kriging of the SAS/KRIGE2D procedure. (v) Finally, adding these estimated values to those predicted by the quadratic trend to obtain the predicted density ($Predlog_{10}SPORES$) in any unmeasured location of the field plot. The Pearson product-moment correlation coefficient between the estimated abundance of ustilospores and the number of ustilospores counted in the cores taken at random not coinciding with those taken systematically, both logarithm-transformed, was calculated with the SAS/CORR procedure to assess the goodness of the estimates.

To test the relationship between the abundance of ustilospores in the soil in winter and the degree of disease incidence in the next season, linear regression analyses were performed between the predicted abundance of ustilospores in the soil and the proportion of diseased plants observed at the end of the following growing season in the quadrats sampled (plants·0.25 m^{-2}). To assess the potential causality or otherwise between variables, that is, to consider the chance of a spurious relationship between them, the spatial dependence of both variables was considered. Multiple regressions were done using the plot width and length coordinates of the plant sampling locations as independent variables, and the residuals from the two multiple regressions were, in turn, analysed by means of linear regression. The purpose of this procedure was to search for the existence of a surface trend, and in the event of a significant relationship being detected between ustilospore abundance and proportion of diseased plants (or density of diseased plants) it will be non-spurious only if the linear relationship between the residuals has a slope significantly different from zero (Fortin & Dale 2005). Besides the proportion of diseased plants, we also use the proportion of partially smutted plants and the diseased plant density as dependent variables. All the mentioned regression analyses were done using the SAS/REG procedure. The predicted number of spores was decimal logarithm-transformed ($Predlog_{10}SPORES$), as was the density of diseased

plants (Log_{10}Disease+1), while the proportion of diseased plants and the proportion of partially diseased plants was arcsine-transformed (ArsinPDis, ArsinPPartialDis) before analyses.

The estimated ordinate intercept and slope of the straight lines relating the logarithm-transformed ustilospore density and the three dependent variables (arcsine-transformed proportions of diseased plants, and logarithm-transformed diseased plant density) were used to draw in a two y-axis graph the relationship between the back-transformed estimated percentage of diseased plants, percentage of partially diseased plants, and diseased plant density (plants·0.25 m^{-2}) with respect to the ustilospore density in a 0.0125 m^3 layer of the upper soil (0.25 m^2 in surface area and 5 cm in thickness).

Moreover, to interpolate the spatial distribution of the disease incidence within the plot with the aim of ascertaining the existence and characteristics of a linear surface trend in its spatial arrangement, analyses of covariance considering the width and the length as covariables were performed on the arcsine-transformed proportion of diseased plants, the proportion of partially smutted plants, and the decimal logarithm-transformed diseased plant density. The Type III sum of squares was computed using the SAS/GLM procedure, and contour plots of the interpolated values were obtained for both variables. The contour plots can help to compare the estimated spatial arrangement of the ustilospore density, obtained by ordinary kriging, with those of the two infected plant phenotypes observed.

Results

All the soil cores taken, without exception, contained *U. syntherismae* ustilospores, but 36.7% of the cores did not contain any caryopsides of *D. sanguinalis*. The mean number of ustilospores estimated in a 5 cm deep soil with a surface area of 0.25 m^2 was $2.6 \cdot 10^6$, ranging from $10.6 \cdot 10^4$ to $10.8 \cdot 10^6$. The number of caryopsides in the same volume of soil ranged from zero to $2.8 \cdot 10^3$, giving a mean number of 479. It is noteworthy that, considering the methodology employed to trap the ustilospores, it is possible that the abovementioned result is an underestimation, while in the case of the number of caryopsides it is not. However, the 2013 *D. sanguinalis* population had, at the end of the growing season, a mean density of 60 pl·0.25 m^{-2}, ranging from 13 pl·0.25 m^{-2} to 172 pl·0.25 m^{-2}, which probably indicates that the 1.5 cm diameter and 5 cm length core was not the most appropriate sampling unit to estimate the caryopsis density in the topsoil.

In spite of these considerations concerning the sampling units employed, looking at the statistics that characterize the sampling strategies (Table 1) it seems clear that neither the spores nor the seeds had

a uniform numerical distribution, and also that the spores were more clumped than the seeds. Firstly, although the variance-to-mean ratio of seeds was higher than 1 (expected value in the random spatial arrangement), this ratio was three orders of magnitude greater for spores than for seeds. Secondly, the differences between the goodness of fit of Poisson and negative binomial probability distributions were very large in the case of spores, while they were relatively small for seeds; the deviance to degrees of freedom ratio would be 1 if the fits were very good. Although both the spores and the seeds fitted the negative binomial probability distribution better, which would indicate a certain level of aggregation, the distribution of the seeds was not very far from the fit to Poisson.

The estimated dispersion parameter of the negative binomial probability distribution gave complementary information that pointed in the same direction, because it was closer to zero for seeds than for spores; if it were exactly zero it would not have overdispersion and the best fit would be with a Poisson distribution. The systematic sampling method gave the most aggregated view of the distribution for both the spores and the seeds, because it had the worst goodness of fit to the Poisson probability distribution and the highest values of the dispersion parameter of the negative binomial. The random sampling method gave a less aggregated perception of the distribution of both propagules.

The seeds correlograms (Fig 2) show levelling of values near to the expected values of non-autocorrelation (Moran's *I* close to zero) or non-significant *P*-values for Moran's coefficient under the null hypothesis that the sample values are not autocorrelated, which is characteristic of absence, or non-detection, of spatial autocorrelation. In turn, the spore correlograms show a trend towards high coefficient values at short distances and low ones at large distances, without achieving non-autocorrelation even in the greatest distance class, which indicates the existence of a gradient in ustilospore abundance.

Both the omnidirectional (not shown) and the four experimental directional semivariograms of seeds (Fig 3) turned out to be pure nugget effect variograms (flat ones) in which semivariance does not increase with the separation distance, indicating isotropy and absence of a spatial structure at the scales at which the observations were made; thus, the absence of spatial correlation makes it impossible to estimate the location of the seeds in the plot. Considering all the analyses done with this variable (SEEDSc), both using the random sampling and using the systematic sampling, we must conclude that, although there is evidence of some degree of aggregation (Table 1), we cannot describe any spatial pattern other than random (Figs 2 and 3), possibly due to the sampling unit employed.

On the other hand, the experimental omnidirectional variogram of spores (not shown) is clearly linear, autocorrelation being present throughout the entire extent of the plot sampled, an indication of surface trend, as has also been indicated by the correlogram (Fig 2). The directional variograms performed with the residuals of the analysis of covariance (Fig 4), which remove the trend, showed that there was no anisotropy in their spatial arrangement, in spite of the fact that the machinery passes were done in the same orientation and direction throughout almost the whole plot. The mentioned analysis of covariance shows that neither the width nor the length were significant sources of covariance at P=0.05, but their quadratic forms did present it (at P=0.001). The best model to fit the semivariance of the detrended observations was the power-sine hole, having the following parameters: nugget=0.055, power slope=$1.4 \cdot 10^{-5}$, power exponent=2.00, sine hole scale=0.027, and sine hole range=4.89. This modelled omnidirectional variogram (Fig 4) has a smooth hole at 10 m, probably indicating periodicity in the patchiness. The hole effect occurs under periodical correlation structure, something that the correlogram did not show previously (Fig 2), but the model revealed the existence of patches hidden by the surface trend.

The contour map of ustilospore abundance (Fig 5) shows a trend with the highest ustilospore density at the SE corner of the plot and the lowest at the NW corner; the gradient of ustilospore density went, broadly speaking, diagonally across. The procedures performed to predict the abundance of ustilospores in the entire plot allowed an acceptable result, because the correlation analysis between the predicted $Predlog_{10}SPORES$ and the $log_{10}SPORES1/3c$ counted in the random samples produced a Pearson correlation coefficient value of 0.818 (n= 37, P<0.001).

On average there were 23.1 diseased plants per quadrat (pl·0.25 m^{-2}) at the end of the 2013 growing season; the mean percentage of diseased plants was 31%, ranging from zero to 64.4%. Of all the diseased plants, the mean percentage of partially diseased ones was 4.2%, ranging from zero to 22.8%. The spatial arrangement of the proportions of disease and density of diseased plants followed linear trends (Fig 5) that showed, as happened in the case of the predicted ustilospore abundance, the highest values at the SE corner of the plot and the lowest at the NW corner. In all three variables analysed the width and the length of the plot were significant covariables (P<0.05).

The linear relationship calculated between the logarithm-transformed estimated abundance of ustilospores and arcsine-transformed proportion of diseased plants at the end of the next growing season has a significantly positive slope (Table 2). When the residuals were linear-regressed the straight line

showed a slope significantly different from zero at 10% probability level (P=0.09). The same behaviour was observed if the dependent variable was the arcsine-transformed proportion of partially diseased plants. When the procedures were repeated searching for a relationship between $Predlog_{10}SPORES$ and logarithm-transformed density of diseased plants, both the slope of the linear regression between the two variables and the slope of the linear regression between the residuals were highly significantly different from zero. Thus, there was potential causality between the variables that describe the disease incidence and the ustilospore density, and their relationship was not attributable only to their spatial distribution.

In summary, where there were higher estimated ustilospore densities there were higher diseased plant densities and larger proportions of diseased plants, because the estimated slopes of the linear regressions were positive (Table 2). Each of these three variables, in turn, were significantly related to the width and length coordinates of the plot, that is, displayed a spatial arrangement within the plot related to the two spatial dimensions. Figure 6 shows the variation in the proportions of diseased plants and in the diseased plant density as a function of predicted ustilospore density, back-transforming the variables and considering the number of soil cores (5 cm in depth and 1.5 cm in diameter) in a quadrat with a surface area of 50 cm x 50 cm. The greyscale bar in the figure indicates the range of predicted ustilospore density of the contour map (Fig 5). The density of diseased plants in the population increased linearly when the density of inoculum increased, whereas the increases in the proportions of diseased plants with the density of inoculum were non-linear. Specifically, the values of proportion of disease change from zero to up to 30% when the ustilospore density varies from the minimum value to $2.0 \cdot 10^6$ ustilospores$\cdot$0.25 m^{-2} (Fig 6), with estimations of ustilospore density obtained in approximately a third of the field (Fig 5).

Discussion

The densities of ustilospores and spikelets observed in winter 2012, evidently conditioned by the sampling strategy (Nicot *et al*. 1984; Ekschmitt & Griffitts 1998; Rossi & Nuutinen 2004), were a reflection of the spatial arrangement of the 2012 (or previous) diseased and non-diseased subpopulations of *D. sanguinalis* distorted by the machinery passes carried out in the agricultural preparation of the soil.

At the time of soil tillage, most of the *D. sanguinalis* plants did not remain erect, but lay on the soil surface, entwined with the debris of the same or other plants. Thus, the majority of dry, well-formed spikelets were not attached to the raquis of the inflorescences, but had fallen close to the mother plant, as has been observed in other grasses (Cheplick 1998). In turn, the majority of *U. syntherismae* sori,

enveloped by the withered leaf sheath, are components of this kind of stubble formed by the dead plants of the community. Our results indicate that the spikelets were distributed with a certain level of aggregation (Table 1), but spatial correlation of data was not detected (Fig 2, Fig 3). It is possible that the lack of autocorrelation could be due to an effect of the machinery pass, dispersing vertically and horizontally the aggregates of spikelets formed in hypothetical clumps of healthy plants. To our knowledge, this lack of autocorrelation is unlikely to be due to intrinsic causes alone, because the *D. sanguinalis* spikelets are hairy; the presence of hairs causes the caryopsides to detach together, forming groups, at least in some individuals.

The ustilopores were spatially arranged in a single patch (Fig 2, Fig 5), following a gradient of abundance that could be the result of at least two things: first, in the preceding season there could have been more diseased plants in the southern half of the plot than in the northern half, and second, the machinery passes could easily drag the plants with sori horizontally, spreading them forward and sideways, enriching the eastern half with ustilospores. Although it is known that smut fungi use several dispersion systems (Piepenbring *et al.* 1998), in this population most of the sori remain quite enclosed by the uppermost leaf sheaths (Vánky 1994) when the plants decay (Verdú & Mas 2015). They must be buried in the soil by the chisel tillage operations, at a depth of no more than 7 cm (Schneider *et al.* 2006), while the forward and the lateral displacement with this plough must be 0.2-1.3 m and 0.25 m respectively (Liu *et al.* 2010). No information is available yet about other dispersal mechanisms of *U. syntherismae* ustilospores, although because we observed that few diseased plants had the mass of spores unprotected before dying, with the smutted raquis above the uppermost leaf, it is reasonable to expect that some spores were wind dispersed over long distances, outside the plot, while others could reach the stubble by means of rain-wash or rain-splash, and so remain in the plot relatively near its diseased plant. These two ranges of dispersion – achieved over short distances here as a result of tillage or rain, and over long distances as a result of wind dispersal – have been pointed out by Piepenbring *et al.* (1988) as a general strategy of smut fungi. Neither is anything known yet about the longevity of *U. syntherismae* ustilospores, but in the literature the ustilospores of some other species such as *U. maydis* are recognized as long-lived, particularly in temperate regions, where they are capable of surviving in wet soil for many years (Christensen 1963). Therefore, the spatial arrangement of ustilospores in the soil may reflect not only the disease expression of the previous season, but also the consequences of some years of sorus production and tillage operations.

Everything we have just said leads us to think that chisel ploughing tillage moves seeds and spores, mixing and placing them in the soil profile at a shallow depth. In fact, a large proportion of these buried large crabgrass seeds could germinate and emerge if environmental conditions were appropriate, according to the available knowledge about movement of weed seeds depending on tillage implements (Rew & Cussans 1997; Grundy *et al.* 1999; Mohler *et al.* 2006; Colbach *et al.* 2014).

The quantitative relationship found between the soil density of the ustilospores and the percentage of diseased plants at the end of the next season was clearly associated with the spatial arrangement of both parameters in the field (Table 2, Fig 5). To summarize, there were some zones of the field in which higher spore densities coincided with higher percentage of disease, and there were other zones in which lower levels of both coincided. It is notable that the tendency is similar for both phenotypes observed, completely and partially smutted plants. For partially smutted plants the relationship is weak; the slope is significantly different from zero, but the coefficient of determination is low (Table 2). Thus, the possibility of spatially heterogeneous selection of these two phenotypes must be ruled out. These spatial distributions lead us to think that there could be field zones that were more favourable than others, both for sorus production and for infection processes, which implied, obviously, seed germination and seedling emergence.

The percentage of diseased plants and the number of sori produced per surface area were clearly plant density dependent in the seasons preceding the soil sampling, being higher when the plant density was higher (Verdú & Mas 2015); now it seems clear that this density dependence was not independent of the field zone. As argued before, it is worth highlighting that this spatial pattern may be maintained for many seasons under the current soil disturbance regime. In fact, the type of soil disturbance regime has a primordial role in the disease persistence, either for the horizontal movement of spikelets and spores, or for their burial at depths that encourage seedling mesocotyl elongation and thus enhance infection.

It is not easy to find a simple and robust quantitative relationship between densities of ustilospores and spikelets in the upper part of the soil profile and the degree of disease incidence in the population, or, in the words of Ettema & Wardle (2002), to elucidate the aboveground consequences of belowground spatial patterning. The difficulties could be attributed to methodological aspects, but it is also plausible that the relationship is intrinsically weak, because there are many processes involved between the presence of both propagules in the soil and the development of the disease (Termorshuizen & Jeger, 2008). Although it is reasonable to expect that the disease incidence depends on the number of

ustilospores and spikelets found in the first cm of the soil profile, it is also true that the disease will only be detected if certain conditions, all of them presumably dependent on both the environment and the genetics of the partners, are fulfilled: (i) the soil microsite environment has to be favourable to a more or less synchronous period of ustilospore and seed germination flushes that become each cohort (Mohler & Callaway 1992; Gallart *et al*. 2009; Dalling *et al*. 2011; Verdú & Mas 2015), (ii) the physical distance between the probasidium and the coleoptile or the mesocotyl of the plant seedlings has to be small enough to allow contact, (iii) the seedling has to be susceptible and the hyphae infective, (iv) for the disease to be apparent, the hyphae have to reach the stem tips and form the next generation of ustilospores inside the sorus enveloped by the leaf. Interaction between individuals only really exists and can play a role affecting the genetic structure of both populations if the first two points are fulfilled.

The results show that the density of diseased plants in the population increased linearly when the density of inoculum increased, whereas the proportion of diseased plants with increased density of inoculum did not increase linearly (Table 2, Fig 6). Considering the quadrat sampling unit (surface area 0.25 m^2 and depth 5 cm), the results obtained suggest that once a threshold of 260000 spores is reached in the soil, one diseased plant is expected to be added to the population for each 120000 ustilospore increase. Since the sites with higher ustilospore abundance also presented higher plant density in the next season, the percentage of diseased plants described a curve (Fig 6) that has the steepest slope at the lowest ustilospore abundances. In the field, values of ustilospore abundance under $1.1 \cdot 10^6$ (corresponding to the first eighth of the curve in Fig 6) have been predicted for a little more than a third of the field (Fig 5), while values above $4.0 \cdot 10^6$ are found in less than 14% of the field's surface area.

The results clearly suggest that, in general, a shortage of spores limited disease incidence. But they also reveal that there was a ratio of thousands of spores to each spikelet in the soil. We are still far from understanding the coevolutionary dynamics of the pathosystem, and for this reason we cannot yet discuss whether fluctuating selection dynamics and/or directional arms race dynamics maintain the within-population variation in pathogen virulence and host resistance (Gandon *et al*. 2008; Laine *et al.* 2011; Tack *et al.* 2012). However, in the light of the results, we can understand how important spatial variation is in explaining disease persistence. Two hypotheses can be formulated if we focus on what mechanisms maintain the existence of plant phenotypes susceptible to infection from season to season, which in turn permits the existence of smut:

(1) As there were spores throughout the top 5 cm of the soil, the probability of encounter is the same (or similar) in the entire field, varying each year with the environmental factors that strongly affect seed germination and seedling establishment. But in zones of the field with low plant densities and therefore low intraspecific competition, infected plants could grow more and faster in higher proportions than in zones with higher densities, maintaining the fungus at asymptomatic levels. The spikelets produced by these plants would be genetically susceptible, if we consider that self-fertilization is predominant in this species (Lemen 1980) and we accept the recognized general existence of a genetic basis for host resistance and susceptibility to infection (Laine *et al*. 2011) and, therefore, also the existence of a site × genetic interaction. Thus, asymptomatic plants would represent an important source of susceptible spikelets, which could be spread by tillage operations. The low proportion of asymptomatic plants found in inoculation experiments (Mas & Verdú 2014) is the principal argument against this hypothesis.

(2) However, it is also possible that the spatial structuring occurs over a spatial scale that we have not examined or, to be more exact, that we have distorted artificially. It is plausible that the method employed to disperse soil particles broke the sori or parts of the sori that formed clusters of thousands of ustilospores, which could be larger than the spikelets. Then, if the ustilospores in the soil were arranged not as a more or less dense blanket, but in clusters, the possibilities of contact between hosts and pathogens would diminish drastically. Under this hypothesis, physical contact between host and pathogen could only take place if each germination and emergence coincides in space with a cluster of spores. Because encounter would not take place in many zones of the field, resistant and susceptible plants could coexist over generations. In this scenario, the selection pressure the pathogen exercises by castrating the host would be very different at the two ends of the plot, from SE to NW. At the SE end, higher plant densities coincide with higher production of sori (per surface unit) but lower production of spikelets per plant, while at the NW end we find lower plant densities, lower production of sori and higher production of spikelets per plant (Verdú & Mas 2015). The causality of the linear relationship found between the ustilospore abundance and plant disease density (Table 2, Fig 6) is a solid argument in favour of this explanation. The strong role that disease escape would play under this hypothesis, even in a small space, should be taken into account in other plant-pathogen systems under study.

To advance in the understanding of the coevolutionary dynamics of this plant-fungus interaction, it would be necessary to distinguish between polymorphisms and pinpoint their concrete location in the field.

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Figure 1

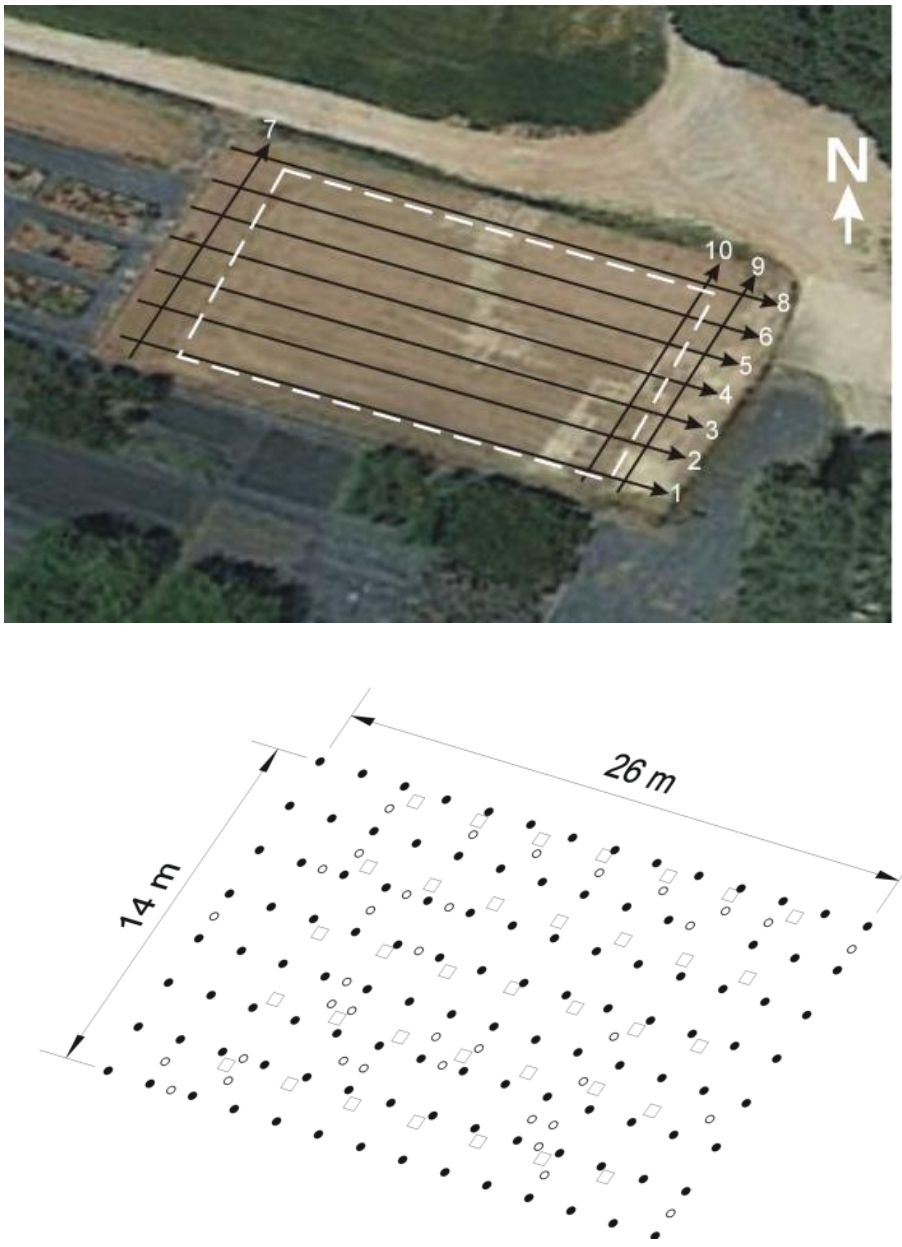

Fig. 1 From top to bottom, scheme of the machinery passes imposed onto a Google Earth view of the study plot located at Torre Marimon (Caldes de Montbui, Barcelona), and soil and plant sampling design. Above, the numbers at the arrows tips indicate the order of the sequence of machinery movements, the broken white line delimiting the sampled surface. Below, locations of the 1.5 cm diameter winter soil samples (● 2 x 2 m grid and ○ random, taken in the middle of the circles), and location of the 0.25 m^2 quadrats during the following summer, drawn to scale.

Figure 2

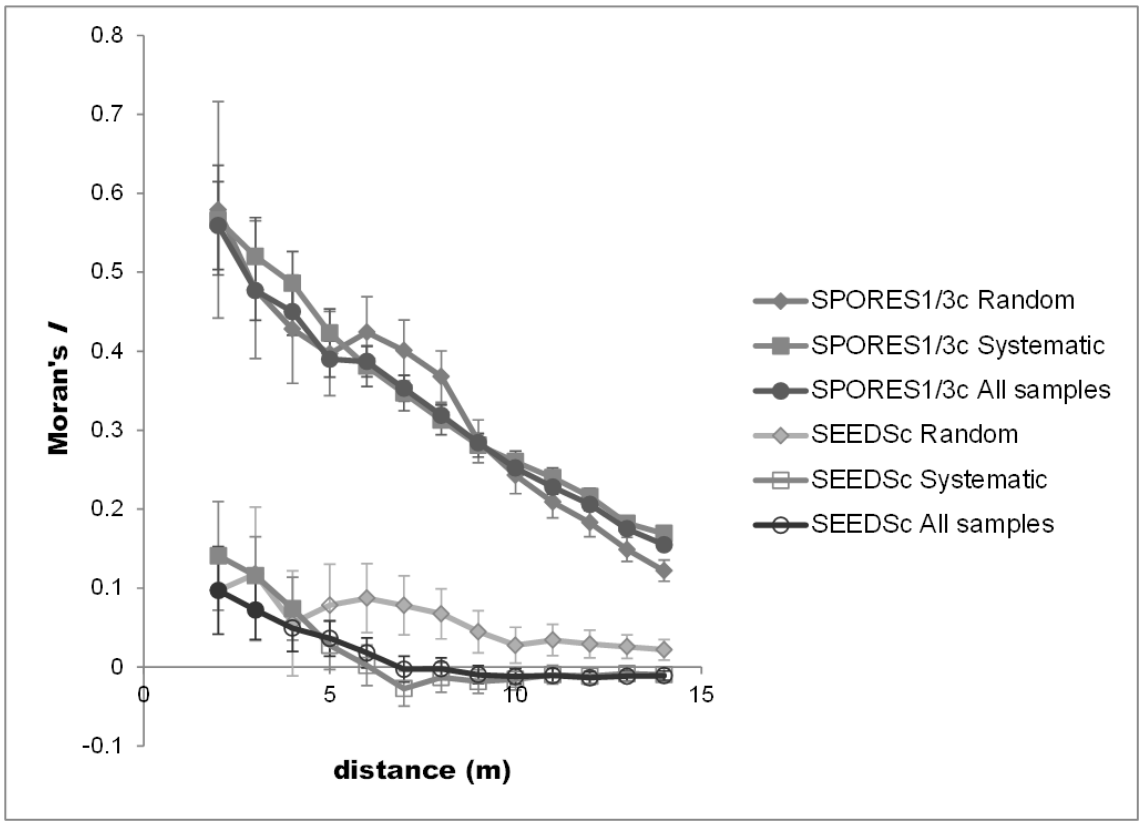

Fig. 2 Moran's *I* correlograms of *Ustilago syntherismae* ustilospore (SPORES1/3c) and *Digitaria sanguinalis* spikelet (SEEDSc) abundance obtained from soil cores sampled at random (n=60), systematically (n=111), and both together. Solid marks indicate significant values at $P<0.05$, while open marks indicate non-significant values; standard deviations are represented by vertical bars.

Figure 3

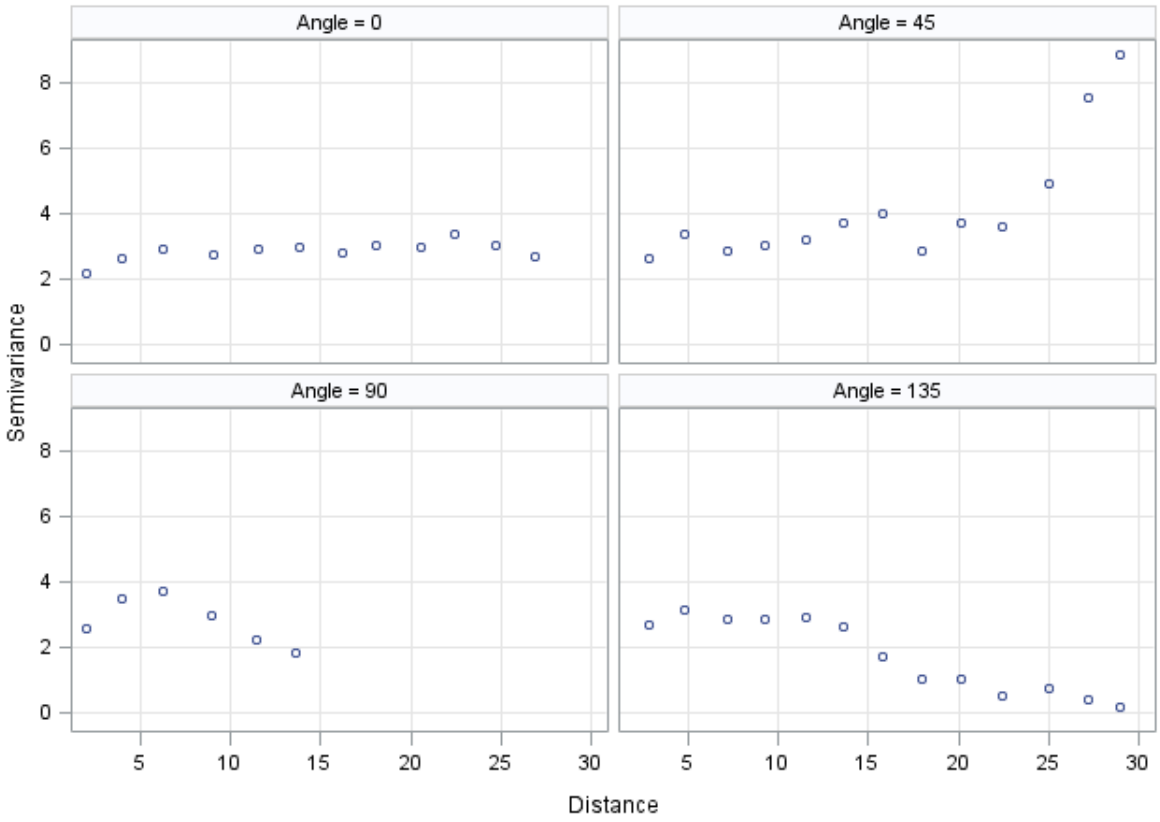

Fig. 3 Four experimental directional variograms of abundance of *Digitaria sanguinalis* spikelets (SEEDSc).

Figure 4

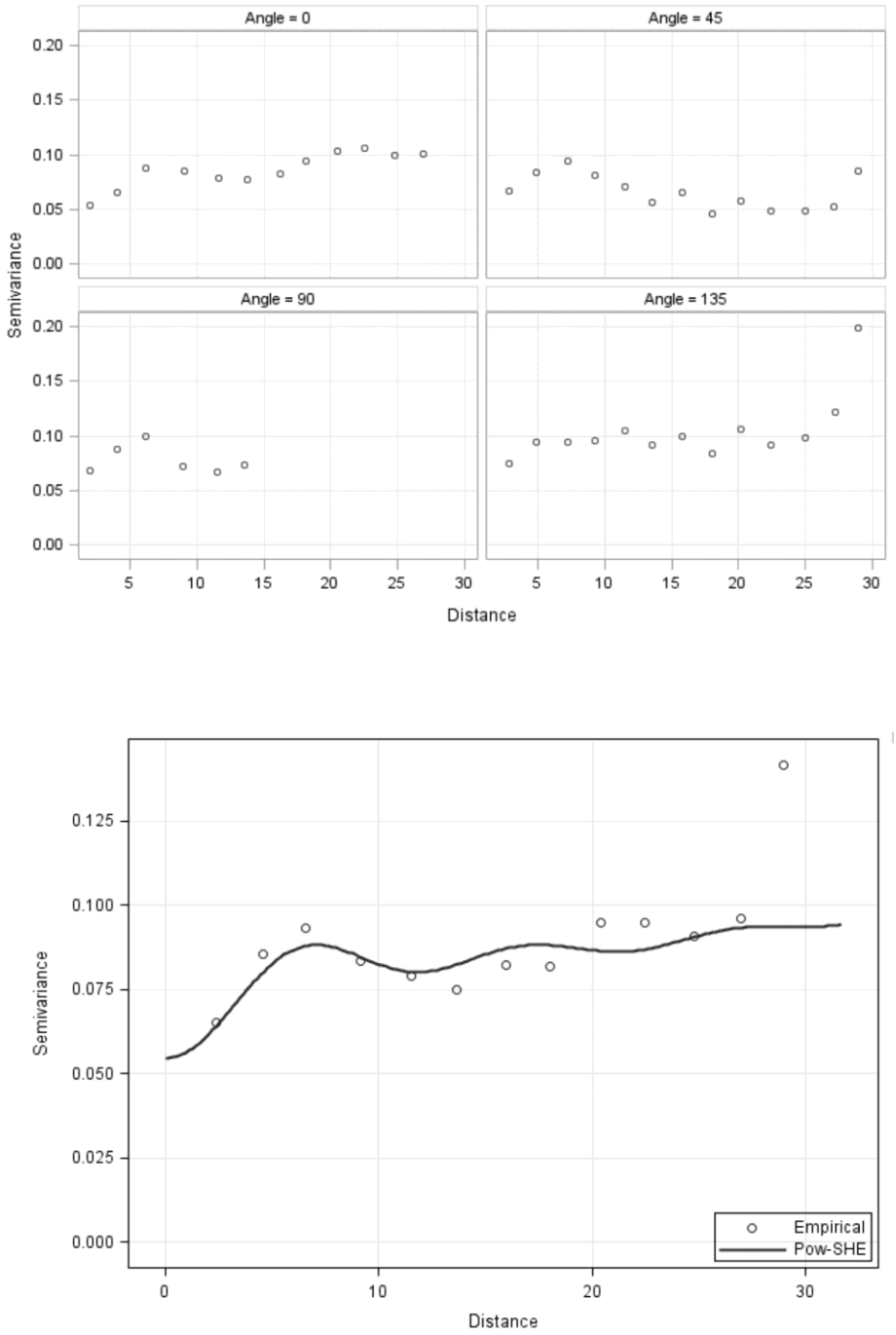

Fig. 4 Four experimental directional variograms (top) and modelled omnidirectional variogram (bottom) of previously detrended abundance of *Ustilago syntherismae* ustilospores (log10SPORES1/3c).

Figure 5

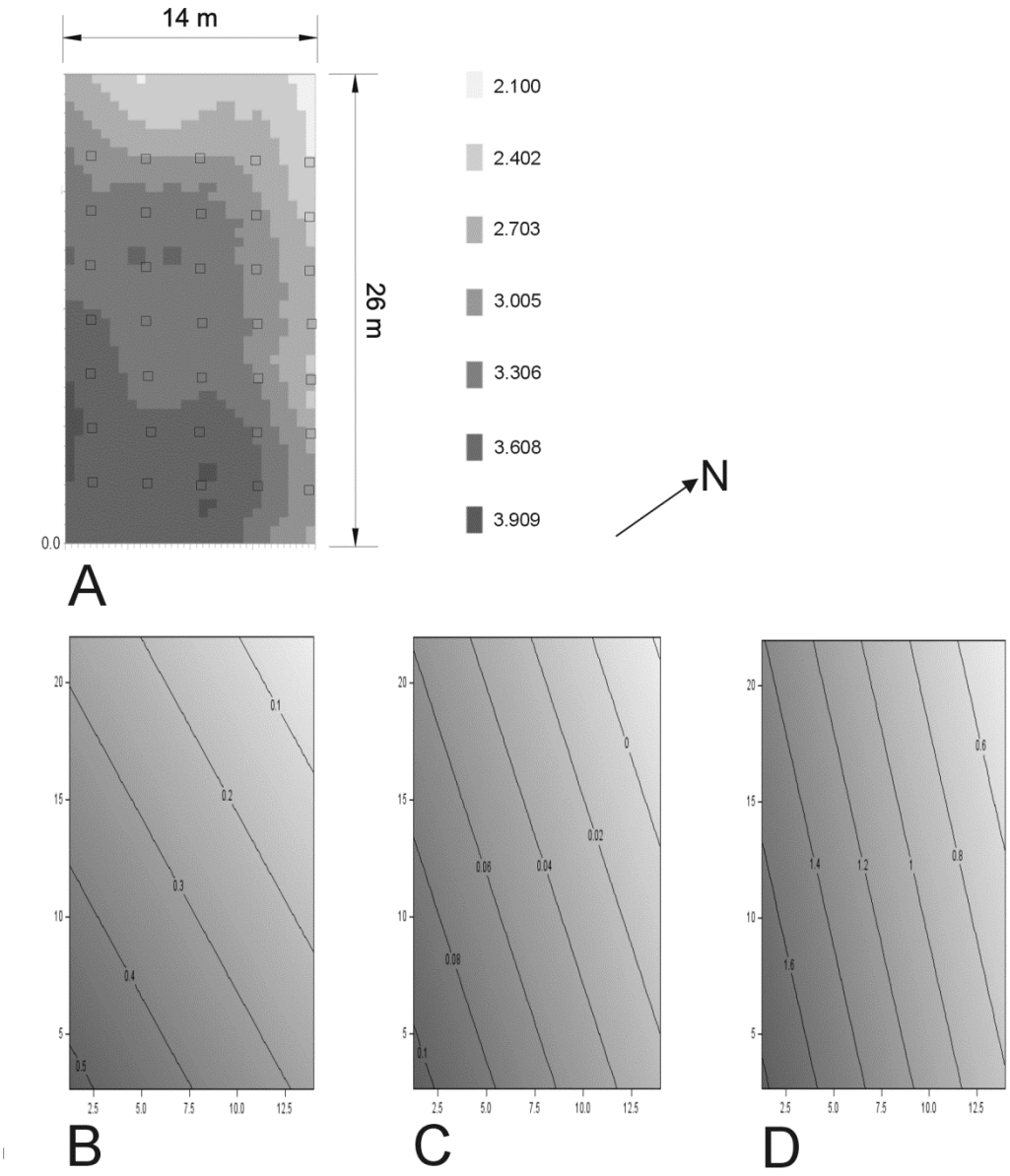

Fig. 5 Contour maps. Map A shows predicted soil ustilospore abundance (decimal logarithm-transformed) in winter 2012. Quadrats sampled in the next season (2013) are shown superimposed (drawn to scale). Maps B, C, and D show the linear surface trends of the following variables: B. proportion of diseased plants; C. proportion of partially diseased plants; and D. diseased plant density. Data was obtained by sampling the quadrats at the end of the next season (2013). Proportions were arcsine-transformed, while ustilospore and diseased plant density was decimal logarithm-transformed.

Figure 6

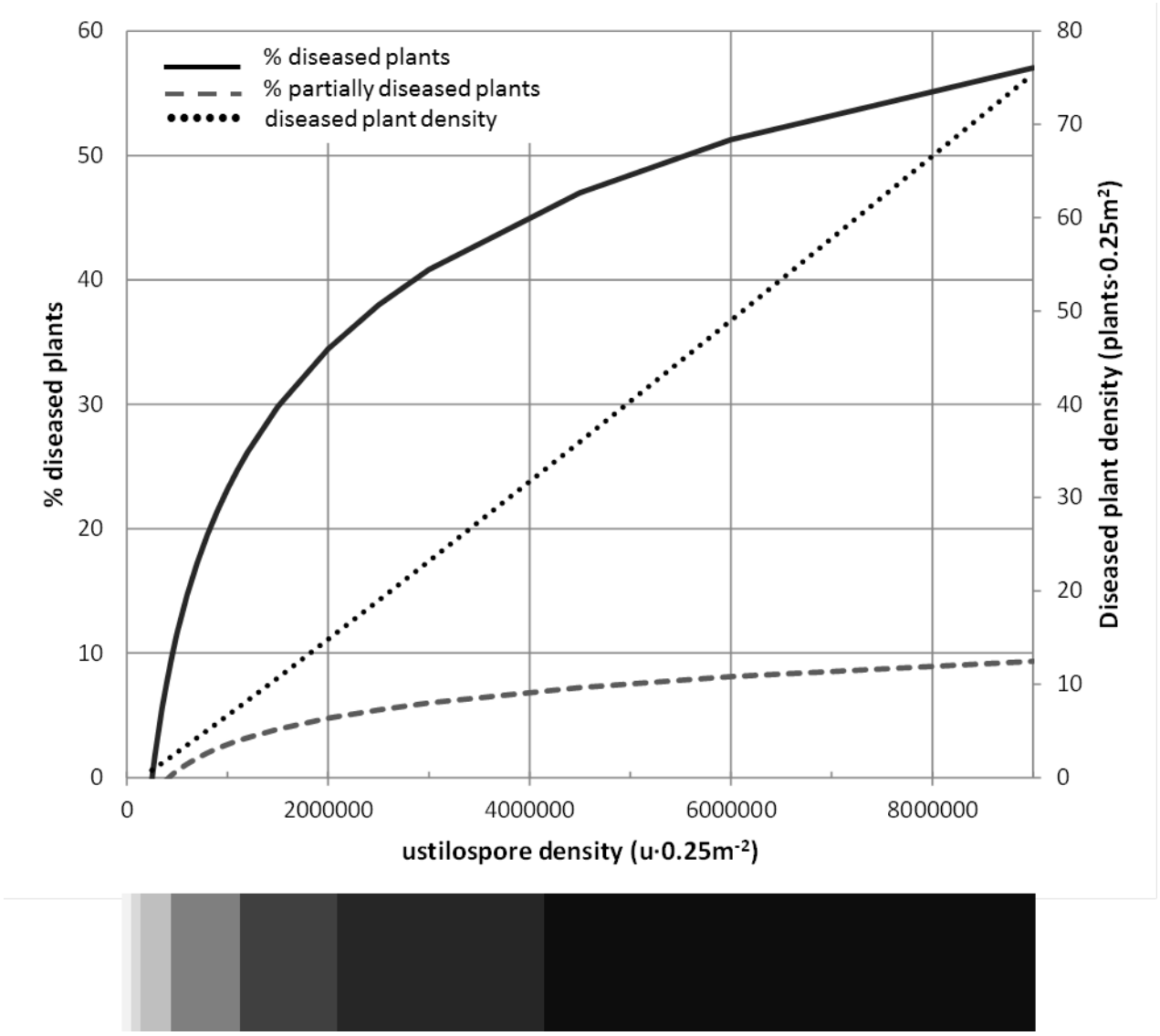

Fig. 6 Variation in the proportions of diseased plants and in diseased plant density as a function of predicted ustilospore density, back-transforming the regressed variables (see Table 2) and considering the number of soil cores of 1.5 mm in diameter in a quadrat with a surface area of 50 cm x 50 cm and a depth of 5 cm. Under the figure, chart with a greyscale corresponding to the intervals of ustilospore abundances in Fig 5.

Table 1

Summary statistics for *Ustilago syntherismae* ustilospores (SPORES1/3c) and *Digitaria sanguinalis* spikelets (SEEDSc) counted in soil cores, varying the sampling method employed and the number of cores considered (n). The first half of the table gives the means, the variances and the variance-to-mean ratios. The second half gives the goodness of fit of the Poisson probability distribution and of the negative binomial probability distribution, as the deviance to degrees of freedom ratio, and the estimated dispersion parameter of the negative binomial fitted function.

	U. syntherismae ustilospores			*D. sanguinalis* spikelets		
	random	systematic	all samples	random	systematic	all samples
Number of cores (n)	60	111	147	60	111	147
Mean	2582.3	2387.2	2450.1	1.47	1.31	1.35
Variance	5523097	5417478	5314588	2.83	2.89	2.64
Variance-to-mean ratio	2138.8	2269.4	2169.1	1.93	2.21	1.95
Poisson[a] goodness of fit (deviance/d.f.)	1904.3	2059.0	1967.0	1.70	2.03	1.84
Negative binomial[a] goodness of fit (deviance/d.f.)	1.15	1.16	1.15	1.07	1.04	1.07
Negative binomial dispersion parameter[a]	0.80	0.99	0.91	0.44	0.90	0.64

[a] Fits to Poisson distribution and to negative binomial distribution were obtained by means of logistic regression using a complementary log link function.

Table 2

Parameter estimates and their levels of significance obtained with three types of analysis: (1) linear regressions between the predicted abundance of ustilospores of *Ustilago syntherismae* and the proportion and the density of diseased *Digitaria sanguinalis* plants (pl·0.25 m^{-2}), considering all diseased plants and solely the fraction of partially smutted plants at the end of the next season, (2) multiple regressions of each of these variables with respect to the width and length coordinates of the plot, and (3) linear regressions between the residuals of the multiple regressions. The predicted number of spores was decimal logarithm-transformed ($Predlog_{10}SPORES$), as was the number of diseased plants in each quadrat ($Log_{10}Disease+1$), while the proportions of diseased plants were arcsine-transformed (ArsinPDis, ArsinPPartialDis).

Type of analysis	Dependent variable	Independent variable/s	Intercept	Slope /Parameter	R^2	n
1	ArsinPDis	$Predlog_{10}SPORES$	-0.93****	0.39****	0.64	35
1	ArsinPPartialDis	$Predlog_{10}SPORES$	-0.18***	0.07****	0.28	35
1	$Log_{10}Disease+1$	$Predlog_{10}SPORES$	-2.23****	1.05****	0.61	35
2	$Predlog_{10}SPORES$	Width	4.08****	-0.06****		
		Length		-0.03****	0.80	35
2	ArsinPDis	Width	0.72****	-0.03****		
		Length		-0.02****	0.66	35
2	ArsinPPartialDis	Width	0.12****	-0.01***		
		Length		-0.001**	0.40	35
2	$Log_{10}Disease+1$	Width	2.00****	-0.08****		
		Length		-0.02**	0.52	35
	Residuals					
3	R-ArsinPDis	R-$Predlog_{10}SPORES$	0.00	0.18*	0.08	35
	R-ArsinPPartialDis	R-$Predlog_{10}SPORES$	0.00	0.20*	0.09	35
3	R-$Log_{10}Disease+1$	R-$Predlog_{10}SPORES$	0.00	1.06***	0.26	35

*P<0.1, **P <0.05, ***P <0.01 and ****P <0.001