Running title: Constraints to smut–plant encounter

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The dynamics of an interaction between *Digitaria sanguinalis* and *Ustilago syntherismae* at local scale is strongly influenced by environment and spatial distribution

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A wild loose smut–summer annual grass interaction was studied to explore the relative importance of some local spatiotemporal patterns of variation for its existence. The prevalence-related variable measured was the proportion of diseased plants (PDP). The mean annual PDP of nine consecutive seasons (2009–2017) was analysed using a generalized linear model with a binomial distribution considering covariables related to rainfall. During the seasons 2013–2015, the precise location of each sample within the plot was taken into account. The PDP of these seasons was analysed in various ways by means of generalized linear models, searching for its spatial variation with plant density in a given season, and with sorus and seeded inflorescence densities of the previous season. Symptomless plants were estimated as 6.1% of the 2015 population. The mean annual PDP ranged from 0.08 to 0.42 and covaried positively with precipitation. Within the field, two zones could be repeatedly delimited among seasons: one in which high plant densities and high PDP co-occurred, and

another with lower values of both in which PDP depended on the sorus density. The role played by differences in the encounter rate within and among seasons is discussed; lack of encounter could be as necessary as encounter for plant–pathogen coexistence over time.

Keywords: infection rate, large crabgrass, loose smut, short-distance dispersal, symptomless plants, within-population variability

Introduction

Studies of interactions between plants and organisms that use plants as nutritional resources have progressed under two coevolutionary perspectives over the past 60 years (Wininger & Rank, 2017); while studies on plant–herbivore interactions focus on an escape-and-radiate approach, plant–pathogen interactions focus mainly on a gene-for-gene approach under arms-race dynamics, where host and pathogen genotype frequencies oscillate over time in attacks and counterattacks.

Recent reviews on wild plant–pathogen associations (e.g. Laine *et al.*, 2011) gather empirical and analytical evidence that maintenance of resistance and virulence polymorphisms could be understood in accordance with either of the two abovementioned perspectives, depending on the particular characteristics of the interaction. Over the last decades, advances in molecular genetics and physiology of both plant and host actors have been made simultaneously with those focusing on populations and community ecology, although, according to Salvaudon *et al.* (2008), with notable exceptions, there has been little communication between the two approaches, aggravated by their differences in level of sampling and types of model systems. However, as Wininger & Rank (2017) point out, conceptual/modelling rather than empirical studies have been used to examine plant–

pathogen coevolutionary dynamics, in contrast with the plant–herbivore studies mentioned above.

Simulations based on mathematical models (e.g. Smith & Holt, 1997; Thrall & Burdon, 2002; Tellier & Brown, 2011; Best *et al.*, 2014; Engelstädter, 2015) have shown the importance of host and pathogen life history traits and spatial structure in disease dynamics, particularly in wild plant–fungal pathogen interactions. However, fungal plant pathogens constitute a very large and heterogeneous group that show enormous diversity in life history strategies and the ways in which they interact with their hosts (Burdon & Silk, 1997; Barret *et al.*, 2008). Lack of information about key aspects of the life histories of both partners strongly limit the usefulness of models developed to understand the dynamics of a particular plant–pathogen interaction. In fact, although many studies on trade-offs between host and parasite based on experimental inoculations, for instance, spotlight the general framework (Laine *et al.*, 2011), the need for more empirical studies is expressed widely in recent papers devoted to simulating and modelling plant–pathogen dynamics (e.g. Tellier & Brown, 2011; Engelstädter, 2015).

In the 1990s, Smith & Holt (1997) published a work that focused on an interaction between annual plants and a particular kind of pathogen, sterilizing fungi, whose ustilospores can infect only during annual grass seedling establishment in a particular environment, that is, with an annual cycle in a temperate climate assuming non-overlapping generations. In their work, conducted within the framework of the use of these pathogens as weed biocontrol agents, they proposed a series of analytical equations that relate the two partners of the interaction, taking into account an increasing number of population parameters and life history aspects. The runs of the last model of the series, the most realistic, showed that the pathogen and the weed can coexist over a wide range of parameter values. The efficiency of infection, or the proportion of smutted plants per year, is a key parameter of all analytical

models devoted to the study of this type of plant–pathogen dynamics, and is assumed to be proportional to plant abundance (Smith & Holt, 1997; Smith *et al.*, 1997), or proportional to seed and spore bank (Smith & Holt, 1997).

With the aim of shedding some empirical light on the causes of a high or low efficiency of infection, this study presents a particular case of a loose smut–summer annual grass interaction, *Ustilago syntherismae–Digitaria sanguinalis* (large crabgrass), in which ustilospores and spikelets overwinter in the soil, infection can take place at an early seedling stage, the fungus is biotrophic and the disease is monocyclic, with only one cycle of both partners each year in a Mediterranean climate (Mas & Verdú, 2014). The symptoms of disease are only visible when the plants are mature, their inflorescences being transformed into sori enclosed in the upper leaf sheath (Vánky, 1994). Healthy and smutted large crabgrass plants were observed in an arable field near Barcelona between 2004 and the present (2019) and, surprisingly, although in the surrounding fields there were *D. sanguinalis* plants each summer, no plants infected by *U. syntherismae* could be found outside that field.

As Burdon & Thrall (2014) argue, the scale at which a reciprocal response with patterns of infectivity and resistance occurs depends on life-history attributes of the system, e.g. mating system and dispersal. Therefore, if that field was viewed as a patch, assuming that the majority of the propagules of both species are formed and dispersed inside it, the plant and the pathogen populations should coevolve during these years within the field. In relation to this, several levels of phenotypic qualitative and quantitative plant resistance have been described (Mas & Verdú, 2014; Verdú & Mas, 2015, 2019), although the variability of fungal infectivity and aggressiveness remains less explored (Jorba *et al.*, 2015). However, even though this variability occurs, and accepting the recognized general existence of a genetic basis for both host resistance and susceptibility to infection (Laine *et al.*, 2011), those plants that form ustilospores and not seeds cannot transmit their genes to the next generation.

Moreover, taking into account that *D. sanguinalis* has a high level of self-pollination (Lemen, 1980) and that practically no seeds survive after three years of burial in the soil (Masin *et al.*, 2006), the persistence of the disease over the years would probably be unviable unless there were some restriction on contact between the pathogen and the plant, avoiding the local extinction of susceptible lineages. So, in order to understand this coevolutionary scenario it is necessary to consider that spatial or environmental constraints on the germinated ustilospores encountering the seedlings may exist.

Because disease escape could be a reflection of spatial phenomena (Burdon, 1987), Mas & Verdú (2018) explored spatial patterns of the overwintering soil propagules of *U. syntherismae* and *D. sanguinalis* in the field, and concluded that ustilospore abundance showed a surface trend that overlapped with a trend in the proportion of diseased plants the following summer. However, because they also found that there was a minimum ratio of thousands of spores to each spikelet in the top 5 cm of the soil, it is still unclear what processes in the dynamics of the interaction could be crucial to enhance or reduce the chance of contact.

The main purpose of the present work is to explore how the proportion of diseased plants, that is, the apparent efficiency of infection, could be relatively affected by some spatiotemporal patterns of variation in a local population of *U. syntherismae–D. sanguinalis*. Specifically, variation in precipitation among seasons, and within-season spatial variations in plant density, sorus production and seeded inflorescence production are considered. Another objective was to quantify the abundance of symptomless plants in a season.

Materials and methods

Study site

The research was performed in the period 2013–2017 on a 15 × 30 m 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, 02°10′17″E). Some data from the period 2009–2012 obtained in the same study plot (Verdú & Mas, 2015) were also used. The plot is surrounded by crops except for a forested patch to the southeast; the crops are adjacent to the plot on the northwest and southwest sides, but there is an unsurfaced road delimiting the plot to the northeast and southeast. 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 is an Inceptisol sandy loam Calcixerollic Xerochrept located on an alluvial terrace with carbonated alluvial deposits as parent materials. Textural data were obtained of two composite samples of the top 5 cm of the soil taken at both the north and south corners of the plot at the end of the experiment, in order to know whether or not there were differences in soil water holding capacity. The field was under crop production until 2006, when the study of the *D. sanguinalis–U. syntherismae* interaction was started. 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. Other details of the history and management and plant communities of the field can be found in Mas & Verdú (2018).

Plant sampling

In each of the five years, population tracking started immediately after the spring soil disturbance. Permanent quadrats, each measuring 0.25 m^2, were distributed in each season in

regular 3 × 3 m grids, but their number and exact position in the field varied from one year to the next (Fig. 1). In 2013 the whole plot was sampled with 35 permanent quadrats placed at intervals of 3 m along five transects 3 m apart (Mas & Verdú, 2018). In 2014 and 2015, the transect contiguous to the edge of the unsurfaced road to the northeast was discarded, and then the field was surveyed by means of 28 quadrats, distributed in four transects, the location of which approximately coincided in the three years (2013–2015). The precise location of each quadrat within the plot was obtained by measuring the distances from one of the vertices to two reference points using a Leica DISTOTM Plus laser distance meter. A few quadrats used in 2013 coincided in location with those of the 2012 sampling (Verdú & Mas, 2015). After that, in 2016 and 2017, the sampling area was extended a few metres on all four sides, with 40 quadrats distributed in five transects (Fig. 1).

After the first flush of *D. sanguinalis*, seedlings of other plant species that appeared within the quadrats were removed weekly, and only *D. sanguinalis* was allowed to grow within them. Except in 2015, an extremely dry summer, almost 90% of the emerged plants survived until the first frosts of autumn (Gallart *et al.*, 2010). At the end of the annual cycle the surviving plants in each quadrat were collected, counted, and sorted according to their disease status. Four disease statuses were considered based on the external signs of the disease in the individuals: (i) completely smutted plants: only sori produced; (ii) partly smutted plants: signs of infection, some sori, but also inflorescences with apparently viable seeds; (iii) non-smutted plants: seeded and apparently disease-free, no signs of infection; and (iv) non-mature plants: plants not flowered and therefore no signs of disease. 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.

In 2013 and 2014, the collected mature plants were frozen and kept in the laboratory to count the number of inflorescences bearing spikelets and the number of sori per plant as

indicators of both fungal and plant fitness. Sorus density (number of sori per 0.25 m^2) and seeded inflorescence density (number of seeded inflorescences per 0.25 m^2) were calculated, both considering all smutted and/or seeded plants and subtracting sori and seeded inflorescences of partially smutted plants. These data were also collected in the 2012 sampling (Verdú & Mas, 2015).

In addition, because the existence of symptomless plants was detected in a forced-infection experiment (Mas & Verdú, 2014), random subsamples corresponding to 20% of the seeded plants of each quadrat collected in 2015 were also frozen and kept to observe the presence or absence of mycelium inside the plant. Sections were made of the basal stem zone by hand under a stereomicroscope, using razor blades, and were not previously embedded in resin. The sections, between 5 and 20 µm thick, were cleared by immersing them in 10% KOH at 45 °C for 2 h, washed with distilled water, stained for 1 min with 0.05% toluidine blue, washed again, and mounted in diluted polyvinyl alcohol for microscopic examination.

Throughout the five growing seasons, as for the seasons 2009 to 2012 (Verdú & Mas, 2015), daily temperature and rainfall data were obtained from a meteorological station that was located 200 m from the field (Station X9, Network of Automatic Weather Stations, Generalitat de Catalunya).

Data analysis

Inter-annual variation

The overall annual proportion of diseased plants, using data from 2009 to 2017, was analysed by means of a generalized linear model with a binomial distribution, searching for its covariation with environmental variables of the seasons. Considering that the infection

process can take place between germination and seedling emergence, variables that could be related to the germination stimuli (such as number of days with precipitation events) and/or the soil water content before and during the major emergence flushes were checked. Each variable was used as a single explanatory variable in a separate model. In addition, the covariation of the annual mean proportion of diseased plants with the logarithm-transformed mean annual plant density ($\log_{10}$ plants m^{-2}) was also analysed. In each of the analyses, performed using the SAS/GENMOD procedure (SAS, 2013), parameters were estimated using logit link function and type III analysis options; the dispersion parameter was estimated as the deviance divided by its degrees of freedom because of overdispersion, and all statistics were adjusted appropriately. Likelihood ratio statistics were used to compute the significance of the source of covariation.

Within-year variation and plant density

Using data obtained from the quadrats in 2013, 2014 and 2015, the analysis of the proportion of diseased plants (PDP) was performed using a generalized linear model with a binomial distribution, as explained above, but now considering the effect 'year' and the covariable 'plant density' in order to confirm or discard the significance of the covariable 'density', which was found to be very significant in the period 2009–2012 (Verdú & Mas, 2015). Plant density (plants m^{-2}) was $\log_{10}$ transformed prior to the analysis.

Within-year variation and spatial coincidence

In order to explore whether or not there were spatial differences within the plot, using the data from 2013, 2014 and 2015 as for plant density, the analysis of PDP was performed using a generalized linear model with a binomial distribution, as explained above, considering the effect 'year', the effect 'zone', and the interaction between both. The effect 'zone' had six levels (Fig. 1); the study plot was divided into six zones, each of them as similar in surface

area as possible, considering that each contained at least four sampling quadrats each season (2013, 2014 and 2015). In addition, plant density at the end of the season (plants per 0.25 m^2) was analysed by performing a generalized linear model of the negative binomial distribution with log link function by means of logistic regression considering the same effects. The least-squares means of the levels of the effects and their 95% confidence limits were computed using probability values from the χ^2 distribution. The SAS/GENMOD procedure was used to perform generalized linear models and means comparisons.

Aspects of the preceding season

With the aim of studying the relative importance of different aspects of the preceding season in the PDP, the variable was analysed by means of a generalized linear model of the binomial distribution with a logit link function considering the effect 'year', the effect 'area' that can be discriminated using the spatial analysis above, with two levels, and the covariables 'sorus density of the preceding season' (number of all sori per 0.25 m^2), and 'seeded inflorescence density of the preceding season' (number of all seeded inflorescences per 0.25 m^2). The effect 'year' has three levels, 2013, 2014 and 2015. The covariables belonging to the preceding season were measured in 2012, 2013 and 2014; specifically, in 2013 and 2014 they were obtained in 28 sampling quadrats, while data from 2012 (Verdú & Mas, 2015) were measured in 10 co-location sampling quadrats (Fig. 1). The analysis was performed following a nested model: the main effect 'area' was nested within 'year', and the covariables were nested within 'area'. The whole procedure was repeated, subtracting the sori and seeded inflorescences formed in partially smutted plants, using the SAS/GENMOD procedure. In addition, the mean values of the two covariables and their 95% confidence intervals were computed for each area.

Estimation of symptomless plant density

A multiple ordinary regression of the estimated proportion of symptomless plants in 2015 was done using the coordinates of the sampling locations as independent variables to search for the existence of a surface trend. The goodness of fit of the obtained parameters was compared with those of autoregressive error models, from one to four-order models, using the Akaike information criterion. The dependent variable was arcsine transformed before analyses, performed with the SAS/AUTOREG procedure.

Results

Inter-annual variation

The annual mean percentage of smutted plants varied between 0.8% and 42% (Fig. 2). The accumulated precipitation over the whole season explained a significant amount of variation in disease prevalence (Table 1). Although the accumulated precipitation from 1 April to 30 June was almost as significant as the accumulated rainfall of the whole season ($P > 0.05$), the number of precipitation events during the same period of time, which ranged from 13 in 2015 to 36 in 2010, was not significant. The accumulated precipitation from 1 May to 30 June was not significant either. The accumulated precipitation in April, indicative of the water supply of the first cohort of each year, which usually emerges in May, was as important as the precipitation in May and June, the period of seedling emergence and plant vegetative development. The overall results of these analyses of the annual proportion of diseased plants seem to indicate that the amount of water retained in the soil during seedling emergence is crucial and strongly positively related to the success of the infection process. Figure 2 shows the estimated curves of the annual mean proportion of diseased plants as a function of accumulated precipitation over the whole season, ranging from 266.2 in 2017 to 784.9 in

2010, and as a function of the accumulated precipitation from 1 April to 30 June, which ranges from 73.1 mm in 2017 to 203.1 mm in 2010.

It should be noted that the annual mean plant density at the end of the season was not a significant covariable of the mean annual proportion of diseased plants (Table 1).

Within-year variation: density dependence and spatial coincidence

If the within-year variation in plant density throughout the study plot was taken into account, then the proportion of diseased plants (PDP) was strongly related to density (Fig. 3). The two sources considered in this analysis were highly significant ($P < 0.0001$). So, in seasons that differed in many environmental aspects (rainfall amount, temporal distribution of precipitation events, temperatures, etc.), there was a strong relationship between the proportion of diseased plants and the within-population plant density.

The third analysis, which explored if there were spatial coincidences among the zones of the study plot that had a particular relative range of plant densities over three years with their PDP, showed that the zones of the field that had relatively high or relatively low PDP were the same in all three years, independently of the year (and, therefore, of the plant density of the year). Both the effect 'year' and the effect 'zone' were highly significant (Table 2), but the interaction 'year × zone' was not. That is, in all three years there were zones with a higher mean proportion of diseased plants than others, and their relative importance was maintained from one season to another. The mean values and 95% confidence limits of the six levels of the effect 'zone' showed that there was a gradient, but in spite of that, zones 1, 3 and 4 had significantly higher mean values of the variable than zones 5 and 6 (Table 2, Fig. 1). The analysis of plant density showed the same high significance of

the two main effects, while the interaction was also non-significant (Table 2). Moreover, although the means were not ordered in exactly the same way for the PDP as for the plant density, there was a spatial coincidence between these two variables, at least in the extremes: the zones with highest mean PDP were also the zones with highest mean densities, and similarly the lowest means almost coincided (Fig. 4). The textural data of the top 5 cm of the soil were the same in both the north and the south corners of the study plot: 63% sand, 19% silt, and 18% clay.

The role of the amount of plant and fungal propagules dispersed in the previous season

The fourth analysis of PDP showed, as expected, the significance of the main effect 'area', which has two levels: the area with higher mean PDP and also high plant density in a given year (area *H*, composed of zones 1, 3 and 4), and the area of lower mean values of PDP and density (area *L*, composed of zones 5 and 6). Zone 2 was not considered in this data analysis, because it allowed intermediate mean values of PDP but low values of plant density. The effect 'year' was the most significant source of variance (Table 3), a phenomenon that has occurred consistently in all the analyses performed on the PDP (Fig. 3, Tables 2 & 3). The covariable 'density of seeded inflorescences in the preceding season' was not significant ($P < 0.05$), but 'density of sori of the preceding season' was significant ($P < 0.05$). Figure 5 shows the difference in the role of this covariable in the different zones: while the PDP in area *H* hardly varied with the sori density of the preceding season, a positive variation occurred in area *L*.

It is noticeable that, if the covariables are calculated only with the sori counted in completely smutted plants and the seeded inflorescences formed only in apparently disease-

free plants, the sorus density of the preceding season is even more significant ($P = 0.0171$), while the preceding seeded inflorescence density remains nonsignificant ($P = 0.2402$).

Figure 5 shows the variation of the proportion of diseased plants with respect to the sorus density of the preceding season each year and in each area at overall mean values of seeded inflorescence density. The predicted curves for 2013 and 2014 in area *L* were nearly parallel, the range of variation being similar and neither curve intercepting close to the zero-zero coordinates. The accumulated precipitation from April to June was 189.6 mm in 2013, 125.1 mm in 2014, and 74.4 mm in 2015 (Fig. 2). The predicted curve in area *L* for 2015, which was a very dry season, showed much less variation in the proportion of diseased plants, which was clearly lower than the other two years in all the values of sorus density considered.

Estimation of symptomless plant density

Observation of the stem histological sections (Fig. 6) of seeded plants from the 2015 season allowed an estimate that, on average, 6.98% (± 2.06%) of the seeded plants were infected but symptomless. No surface trend was found in the regression analysis of the estimated proportion of symptomless plants of 2015, as neither of the two spatial coordinates was significant ($P > 0.36$ for both). The autoregressive error correction did not make evident any spatial trend either.

Discussion

The mean annual proportion of smutted plants in the field was robustly dependent on the amount of precipitation during the season (Table 1, Fig. 2), revealing the existence of an

interplay between the plant, the fungus and the environment. This idea is not new in wild plant–pathogen population biology (Burdon, 1987), but particular studies that quantify the effects of abiotic environmental factors on disease prevalence at population level are not abundant in the literature (e.g. García-Guzmán *et al.*, 1996; Lebeda *et al.*, 2008; Desprez-Loustau *et al.*, 2010; Penczykowski *et al.*, 2015). Precipitation would be expected to affect the mean annual diseased plant density, because *D. sanguinalis* seedling emergence and survival clearly depends on it, especially during May and September (Gallart *et al.*, 2010). But the present results indicate that germination of *U. syntherismae* ustilospores and/or their encounter with *D. sanguinalis* seedlings will be dependent on an amount of soil water content greater than that needed for seedling emergence, leading to a probable variation in their encounter, as has been found in other biotrophic plant–fungal pathogen interactions (Desprez-Loustau *et al.*, 2010).

Clearly, there was a strong positive relationship between the proportion of diseased plants and the within-population plant density. This was the case not only for the seasons 2013 to 2015, but also for 2009 to 2012 (Verdú & Mas, 2015). In addition to the effects of the amount of precipitation, there could be intrinsic factors, that is, aspects related to the disease dynamics in space and time, that play a role in the within-year variation of the proportion of diseased plants and, at the same time, are related to plant density. Antonovics & Levin (1980) point out that because site effects may be confounded with density effects, the interpretation of the relationship between density and probability of disease requires caution. In this case the infection process per se should not be density-dependent, because the disease is monocyclic (Mas & Verdú, 2014).

Searching for these probable site effects, it was found that the field could be divided into two areas: one with higher densities and at the same time higher disease severity, and another in which both traits are lower. Looking at the results on accumulated precipitation,

the first consideration to explain the existence of these two areas could be related to overlapping heterogeneity in the soil water retention ability, but the results from the soil textural data did not support this idea. However, microspatial heterogeneity affecting the encounter between germinated ustilospores and seeds should not be completely discarded, because the encounter could be mediated by many other environmental factors that have not yet been quantified. This could include the amount of gravel in the topsoil, but particularly shading and edge effects, because shading would keep the soil moist and, moreover, it is known that variations in light stimuli strongly affect the infection process (Mas & Verdú, 2014). During the spring, plot zone 1 and half of zone 3, both belonging to area *H*, were shaded by the margin trees, while the others were not.

Within the populations studied here, the proportion of diseased plants was affected differently by the production of plant and fungal propagules in the preceding season. It could be that in the area with a high mean proportion of diseased plants and at the same time high density (area *H*), the disease incidence in a given season was only limited by the amount of water needed for the germination of both propagules, indicating that the encounter between them was not restricted spatially if germination occurred. On the other hand, the amount of sori in the soil of area *L* limited the infection process; despite the amount of seeded inflorescences produced being similar throughout the plot, area *L* allowed significantly lower mean plant density than area *H* (Table 2), probably due to some environmental factors that limited it more than in the rest of the plot. All these findings are consistent with the soil spatial distribution of seeds and spores described by Mas & Verdú (2018), and reinforce their idea that at the time of germination the ustilospores in the soil were probably already arranged not as a continuum but in clusters, each sorus being a cluster. According to the review by Piepenbring *et al.* (1998) on dispersal strategy of smut fungi, the sori produced by plant decay would be the short-distance transmission units. Burdon & Thrall (2014) argued

that physical environmental differences in soil structure and the degree of exposure of host populations to persistent environmental variables or to drying conditions means that not all host populations are exposed to the same probability of pathogen establishment and survival; the present findings indicate that these differences can take place within local populations.

The chisel tillage operations performed in the field, apart from causing burial at a depth of no more than 7 cm (Schneider *et al.*, 2006), can displace the decayed plants with sori about 0.2–1.3 m forward and 0.25 m laterally (Liu *et al.*, 2010). In turn, lightweight spikelets such as those of *D. sanguinalis* could be horizontally scattered from 0.5 m behind to 4.8 m in front of their initial position (Rew & Cussans, 1997). Therefore, it seems that spikelets could be dispersed over longer distances than sori under the soil disturbance regime, and so spatial differences in sorus density could be preserved more from one year to another than differences in seed density.

However, in the event of there being no differences in the encounter rate between the two areas of the plot, the frequency of symptomless plants might possibly be higher at lower densities than at higher densities, giving a relatively high proportion of smutted plants at high densities. The plastic development of plants is one of the more powerful density-reactive mechanisms, and competition from neighbours may itself influence the ability of a pathogen to grow systemically within a plant (Burdon, 1987). Studies on the distribution of hyphae of biotrophic fungi within plants (Fullerton, 1975; Verdú & Mas, 2019) suggest that certain inflorescences can be non-smutted if the plant has the ability to elongate the internodes or branch faster than the ability of the hyphae to colonize the developing buds. However, because a surface trend of symptomless plant density in the plot has not been found, this possibility should be ruled out.

Specifically, in the 2015 season an estimated 7% of the seeded plants fell within the category of symptomless, which represented 6.1% of the overall plant population. This finding, although less robust than those concerning the other two infected plant phenotypes, is relevant to explain the prevalence of the disease over seasons, because these individuals can contribute to plant fitness, presumably giving susceptible offspring. The partially smutted plants were present at lower frequencies, 3.2% considering four seasons, but because they formed, on average, only one seeded inflorescence per each six sori (Verdú & Mas, 2019), their contribution was mainly to fungal fitness, presumably giving low-infectivity (Jorba *et al.*, 2015) or low-aggressiveness offspring. Meyer *et al.* (2010) also reported the existence of *Bromus tectorum* phenotypes with internal hyphae of *Ustilago bullata* that do not develop the disease.

The literature indicates that qualitative plant resistance, which allows individuals to avoid infection, has a genetic basis governed by interaction loci (Dybdahl & Storfer, 2003; Salvaudon *et al.*, 2008; Best *et al.*, 2014). Around 40% of the *D. sanguinalis* spikelets formed in the 2009 study plot population were estimated to be qualitatively resistant (Mas & Verdú, 2014). The fraction of plants susceptible to infection but quantitatively resistant would reach approximately 10% of the population, the estimated percentage of symptomless plants being added to that of partially smutted plants (Verdú & Mas, 2019). So, in rough numbers, if disease escape did not occur, around 50% of the plants would be completely smutted, but this disease severity was observed only in some particular samples located in area *H* of the field, while on average it was not attained in any of the nine years studied.

Moreover, if the encounter rate were not restricted, considering that *D. sanguinalis* is highly inbred (Lemen, 1980), its soil seed bank viability (Masin *et al.*, 2006), and the sterilizing effect of the pathogen, in a few seasons the only within-field sources of susceptible spikelets would be those formed on symptomless plants, on partially smutted plants, and on

presumably resistant heterozygotes that generate a segregating offspring. Meyer *et al.* (2010), who evaluated the resistance of *B. tectorum* to *U. bullata*, reported levels of heterozygosity of less than 1% on average of almost a hundred populations of the grass. If the level of heterozygosity of *D. sanguinalis* was similar, it can be deduced that, for example in 2013 or 2014, to reach a proportion of diseased plants of up to 35%, at least 25% of the susceptible seedlings would necessarily have been offspring of susceptible plants that would not have had encounter with the fungus during the previous seasons.

All the empirical results presented and discussed here suggest that the encounter rate between the early *D. sanguinalis* seedling and the infective *U. syntherismae* hyphae was far from 100%. The shortage in precipitation during the season was the most important restriction among those studied, and clearly was more relevant than the overlapping spatial restrictions found within the field. However, it can be argued that the low encounter rate paradoxically facilitates the local prevalence of the disease over seasons, ensuring sufficient frequency of susceptible seedlings. The local plant–pathogen interaction studied here would be an example of, in the words of Barrett *et al.* (2008), how the causal relationships between spatial structure, life history and evolutionary dynamics are important traits for determining disease incidence, prevalence and severity. At the same time, the results indicate that caution is needed when interpreting the results of some cross-infection experiments between plants and pathogens from an adaptive perspective.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends

Figure 1 Plot sampling locations of 0.25 m^2 quadrats during the seasons 2013–2017, and also some from 2012 whose data on plant reproductive structures were used, drawn to scale. The broken lines delimited six zones of the plot, from Z1 to Z6, used to explore whether or not there were spatial differences in the proportion of diseased plants within the plot with data from 2013, 2014 and 2015.

Figure 2 Variation in the mean annual proportion of *Digitaria sanguinalis* plants diseased by *Ustilago syntherismae* as a function of accumulated precipitation (mm) between 1 April and 31 October (top), and as a function of accumulated precipitation (mm) between 1 April and 30 June (bottom), in nine years (2009–2017) in a field near Barcelona, Spain. Observed values, labelled with the year number, and fitted logit function with 95% confidence limits are represented.

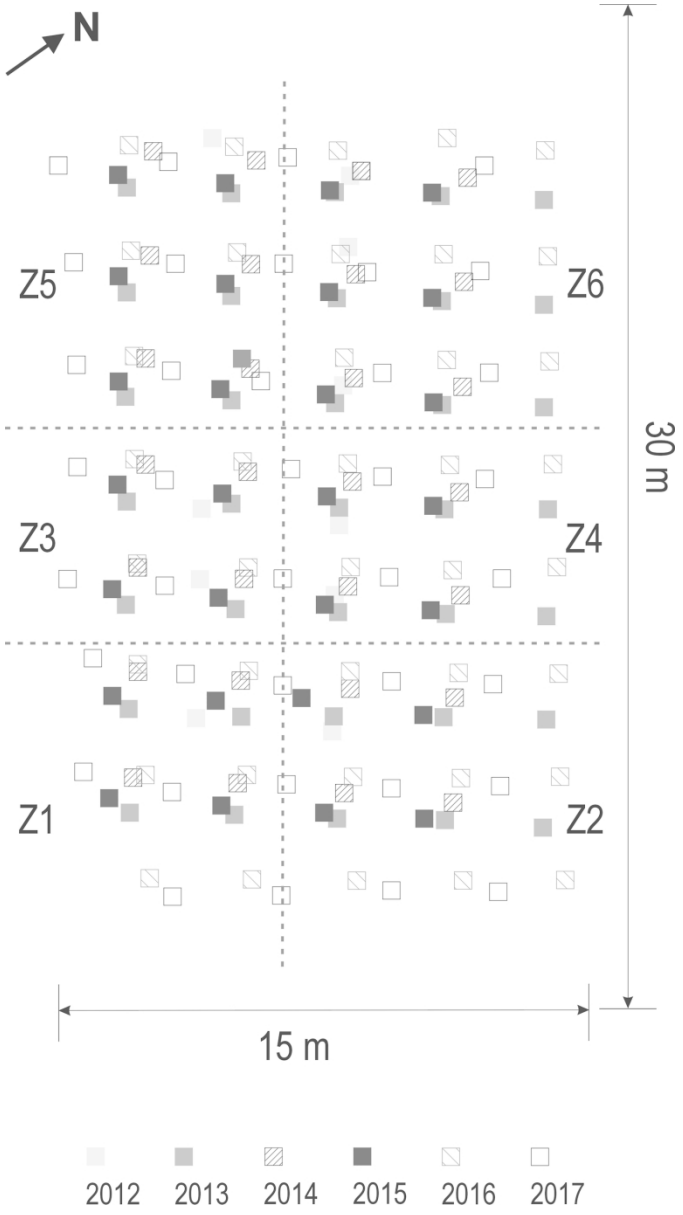
Figure 3 Variation in the proportion of *Digitaria sanguinalis* plants diseased by *Ustilago syntherismae* as a function of within-population density in three years in a field near Barcelona, Spain. Observed values (○, +, ×) and fitted logit functions (lines) with 95% confidence limits from the parameters estimated in the generalized linear model are represented.

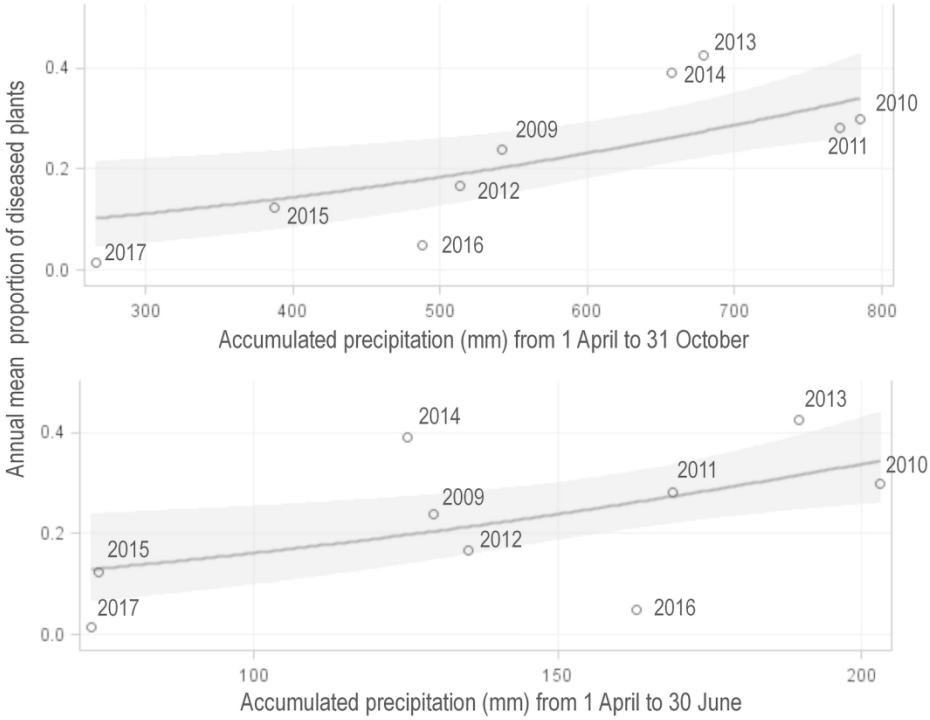
Figure 4 Greyscale plot diagrams representing the spatial variation among six zones (Z1–6) in the proportion of *Digitaria sanguinalis* plants diseased by *Ustilago syntherismae* (left) and in mean *D. sanguinalis* plant density (right) in three years in a field near Barcelona, Spain. Numbers inside the zones are mean values.

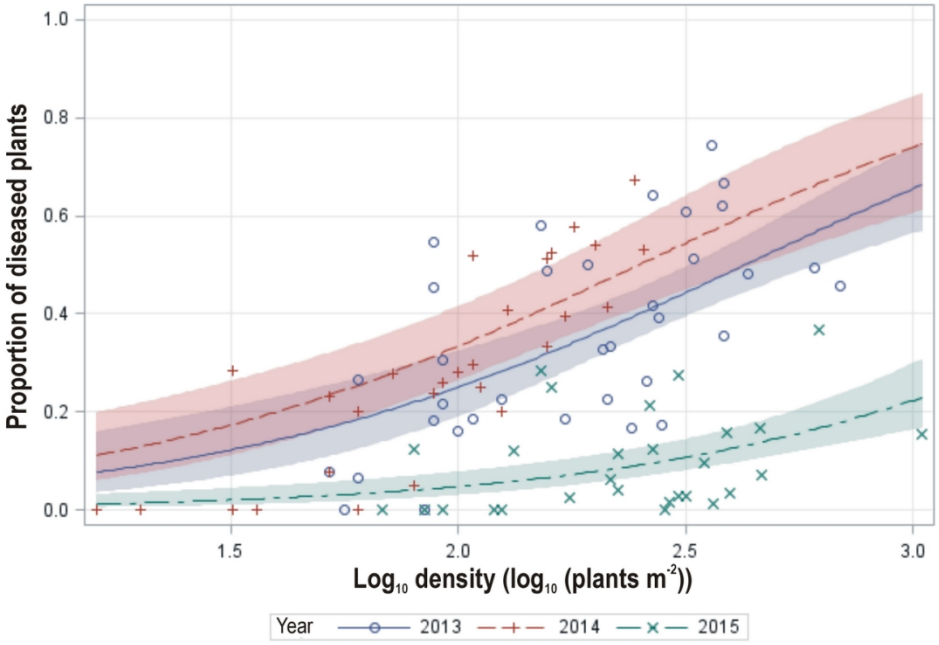
Figure 5 Variation in the proportion of *Digitaria sanguinalis* plants diseased by *Ustilago syntherismae* as a function of sorus density of the preceding season in three years in two areas of the plot, characterized by low and high proportion of diseased plants, in a field near Barcelona, Spain. Observed values (○, +, ×) and fitted logit functions (lines). Fitted functions were computed at mean seeded inflorescence density (366.9 seeded inflorescences per 0.25 m^2).

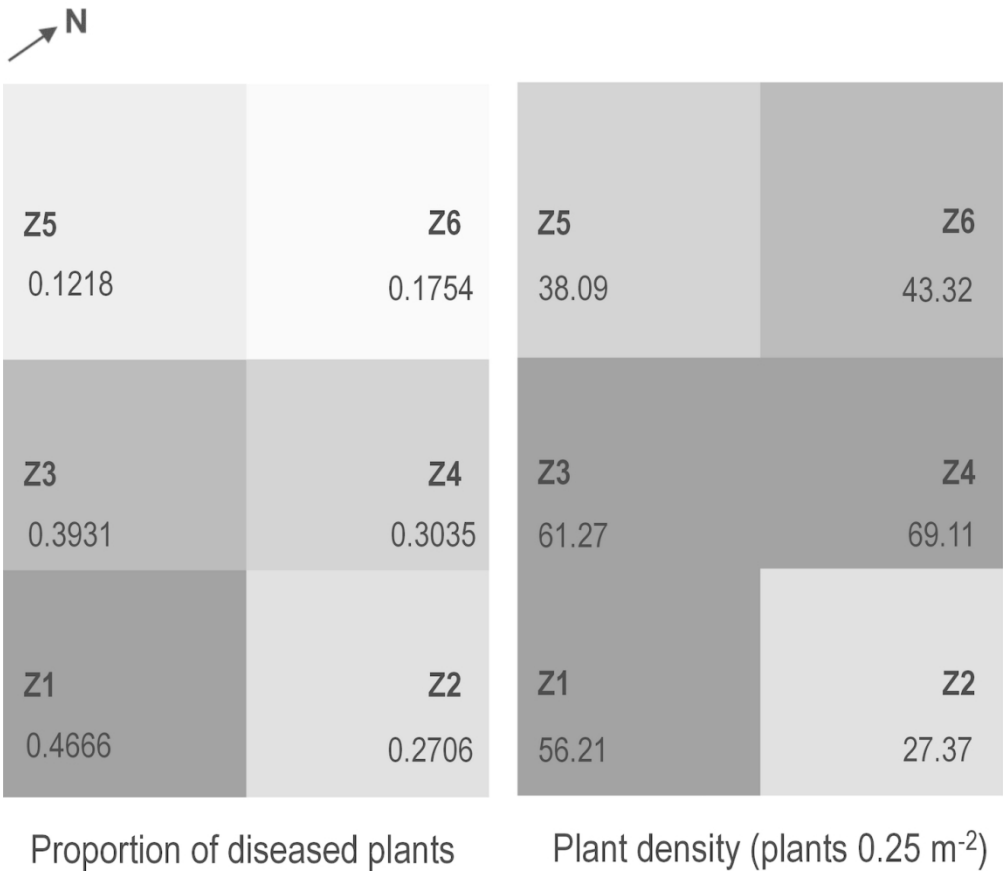
Figure 6 Micrographs of longitudinal stem base sections from *Digitaria sanguinalis* plants infected by *Ustilago syntherismae* from Torre Marimon (Caldes de Montbui, Barcelona, Spain) showing hyphae oriented following the direction of the host vascular bundles: (a) from

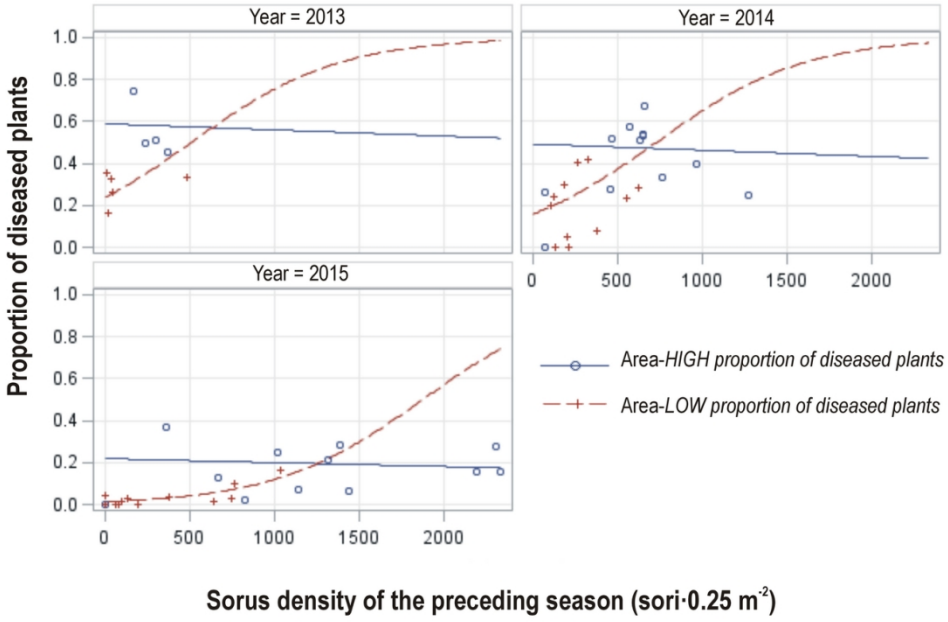
a symptomless plant, (b) from a completely smutted plant; both belonging to the 2015 population.


N
Z5
Z6
Z3
Z4
Z1
Z2
30 m
15 m
2012
2013
2014
2015
2016
2017


Annual mean proportion of diseased plants
0.0
0.2
0.4
300
400
500
600
700
800
2013
2014
2010
2009
2011
2012
2015
2016
2017
Accumulated precipitation (mm) from 1 April to 31 October
0.0
0.2
0.4
100
150
200
2013
2014
2010
2011
2009
2012
2015
2016
2017
Accumulated precipitation (mm) from 1 April to 30 June


Proportion of diseased plants
1.0
0.8
0.6
0.4
0.2
0.0
1.5
2.0
2.5
3.0
Log10 density (log10 (plants m-2))
Year
2013
2014
2015


N
Z5
0.1218
Z6
0.1754
Z3
0.3931
Z4
0.3035
Z1
0.4666
Z2
0.2706
Proportion of diseased plants
Z5
38.09
Z6
43.32
Z3
61.27
Z4
69.11
Z1
56.21
Z2
27.37
Plant density (plants 0.25 m-2)


Year = 2013
Year = 2014
Year = 2015
Proportion of diseased plants
1.0
0.8
0.6
0.4
0.2
0.0
0
500
1000
1500
2000
Area-HIGH proportion of diseased plants
Area-LOW proportion of diseased plants
Sorus density of the preceding season (sori·0.25 m-2)

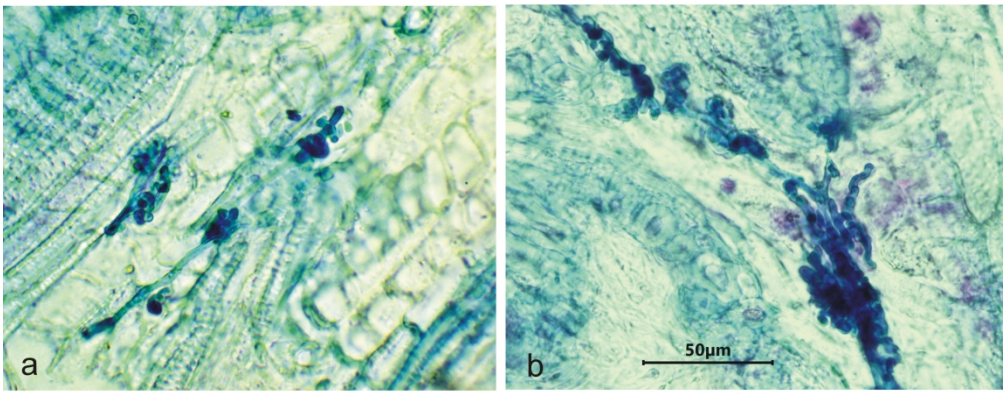

a
b
50µm

Table 1 Likelihood ratio statistics of the analyses of mean annual proportion of *Digitaria sanguinalis* plants diseased by *Ustilago syntherismae*, differing in the source of covariation considered, taking into account data of nine seasons (2009–2017) obtained in a field near Barcelona, Spain

Covariable	d.f. numerator/d.f. denominator	χ^2	$P > \chi^2$
Accumulated precipitation 1 April–31 October (mm)	1/7	8.46	0.0036
Accumulated precipitation 1 April–30 June (mm)	1/7	7.23	0.0072
Accumulated precipitation 1 May–30 June (mm)	1/7	0.96	0.3271
Number of days with precipitation 1 April–30 June	1/7	2.22	0.1361
Annual mean plant density at the end of the season ($\log_{10}$ plants m^{-2})	1/7	0.08	0.7785

Table 2 Likelihood ratio statistics of effects 'year', 'zone' and 'year × zone', in the analysis of proportion of *Digitaria sanguinalis* plants diseased by *Ustilago syntherismae* and of plant density at the end of the season in a field near Barcelona, Spain

		d.f. num./d.f.			Level of Year			Level of Zone		
	Source	den.	χ^2	$P > \chi^2$	Year	LSM	95% CL	Zone	LSM	95% CL
Proportion of	Year	2/72	112.24	<0.0001	2013	0.4315	0.3852–0.4792	1	0.4666	0.3841–0.5509
diseased plants	Zone	5/72	56.67	<0.0001	2014	0.3726	0.3032–0.4476	2	0.2703	0.1786–0.3869
	Year × Zone	10/72	12.94	0.2273	2015	0.1033	0.0724–0.1454	3	0.3931	0.3205–0.4707
								4	0.3035	0.2422–0.3726
								5	0.1218	0.0682–0.2082
								6	0.1754	0.1278–0.2359
					Level of Year			Level of Zone		
	Source	d.f.	χ^2	$P > \chi^2$	Year	LSM	95% CL	Zone	LSM	95% CL
Plant density	Year	2	31.44	<0.0001	2013	57.58	47.65–69.58	1	56.21	41.35–76.40
(plants per 0.25	Zone	5	22.75	0.0004	2014	27.22	22.04–33.62	2	27.37	20.28–36.92
m^2)	Year × Zone	10	9.3	0.5042	2015	66.14	53.93–81.12	3	61.27	45.09–83.27
								4	69.11	51.77–92.24
								5	38.09	29.24–49.62
								6	43.32	34.11–55.01

LSM, least-squares means; CL, confidence limits.

Table 3 Likelihood ratio statistics of effects 'year', 'area', and the covariables 'sorus density of the preceding season' and 'seeded inflorescence density of the preceding season', in the analysis of proportion of *Digitaria sanguinalis* plants diseased by *Ustilago syntherismae* in a field near Barcelona, Spain, performed following a nested model

Source	d.f. numerator/d.f. denominator	χ^2	$P > \chi^2$
Year	2/47	61.24	<0.0001
Area (Year)	3/47	13.07	0.0045
Preceding sorus density (Area) (sori per 0.25 m^2)	2/47	6.72	0.0347
Preceding seeded inflorescence density (Area) (inflorescences per 0.25 m^2)	2/47	2.38	0.3042