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Can the emergence of pine *Diplodia* shoot blight in France be explained by changes in pathogen pressure linked to climate change?

Bénédicte Fabre¹, Dominique Piou², Marie-Laure Desprez-Loustau³ and Benoît Marçais¹

¹UMR1136 Interactions Arbres-Microorganismes, INRA-Nancy, F-54280, Champenoux, France;

²Département de la Santé des Forêts, Pierroton, F-33612 Cestas, France

³UMR1202 BioGeCo, Pathologie Forestière, INRA, F-33612 Cestas, France

Corresponding author: Benoît Marçais, Tel.: +33 383394053, Fax: +33 383394069, Email: marçais@inra.nancy.fr

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Summary

Sphaeropsis shoot blight, caused by *Diplodia pinea* and *D. scrobiculata*, damage conifers throughout the world. In France, the first disease outbreaks were reported during the 90^{ies}. The factors associated with the pathogen presence in stands and the relationship between pathogen and disease distributions were analysed in order to understand the *Sphaeropsis* emergence. Eighty two stands of *Pinus nigra*, *P. sylvestris*, *P. pinaster* and *P. radiata* were visited. Cones were collected on the ground to assess the pathogen frequency. *Diplodia* spp were isolated and determined by a species-specific PCR test. The role of potential explaining factors of *D. pinea* prevalence on cones was analysed by logistic regression. *D. pinea* was the dominant species in visited stands. The main factors influencing the pathogen presence selected in the models were host species (the pathogen being less frequent on *P. pinaster* than on *P. nigra* and *P. sylvestris* cones), winter temperature and summer rain, which were both positively correlated with cone colonization. The climate became more favourable to *D. pinea* presence within the last 15 years compared to the previous 30-year period. By contrast, future climatic changes over the next 40 years should have far less impact on the pathogen presence.

Introduction

Emerging plant diseases, i.e. diseases that either increase in geographical or host range or in severity within their range, are a major issue throughout the world (Harvell *et al.*, 2002, Anderson *et al.*, 2004). Among several causes reported to influence disease emergence, climate change and the introduction of pathogens in new areas linked to the increase in international trade are very important (Anderson *et al.*, 2004, Desprez-Loustau *et al.*, 2007, Sturrock *et al.*, 2011). Several plant pathogens might have increased their range or severity due to improved environmental conditions for disease development (Garrett *et al.*, 2006). Indeed, climate change affects host-pathogen interactions by either direct effects on pathogens or by modifying host physiology and resistance. Direct effect include increased

pathogen multiplication during the vegetation season, for example increase in foliar pathogens such as *Dothistroma septospora* during warmer and moister springs (Woods *et al.*, 2005), or increased pathogen survival in milder winters (Marçais *et al.*, 1996). Indirect effects via altered host physiology include modification of host resistance in different temperature ranges such as in the wheat-*Puccinia recondita* system (Browder & Eversmeyer, 1986) or increased host susceptibility after a drought (Blodgett *et al.*, 1997, Desprez-Loustau *et al.*, 2006). Disentangling the direct and indirect effects of climate change on a plant disease is often difficult because, as the pathogens are usually present only on affected hosts, it is difficult to determine whether an increased frequency is caused by climatic conditions more favourable to the pathogen multiplication or to an increased host susceptibility. While many studies on plant emerging diseases have dealt with the introductions of pathogens in a new area, the impacts of climate change on plant disease remains little documented so far (Garrett *et al.*, 2006, Desprez-Loustau *et al.*, 2007).

Sphaeropsis shoot blight is a good example of emerging disease where several factors could have promoted emergence. This disease, induced by *Sphaeropsis sapinea* (Fr.:Fr.) Dyko and Sutton (syn. *Diplodia pinea* (Desmaz.) J. Kickx fil.), causes serious damage on *Pinus* spp. throughout the world (Nicholls & Ostry, 1990, de Kam, 1985, Burgess *et al.*, 2004). In Europe, the disease was first reported as a major pine problem by de Kam in 1985. In France, severe *Sphaeropsis sapinea*-induced pine declines were reported for the first time in the early 90^{ies} on *Pinus nigra* and *P. sylvestris* (Piou *et al.*, 1991). Although the pathogen had been present in France since at least the end of the 19th century and was commonly observed on pines throughout the country in the 70ties by Morelet (1980) (under the name *Macrophoma pinea*), it was only reported as a minor pine disease (Lanier *et al.* 1976). De Kam (1985) suggested that several factors could concur to the *Sphaeropsis* shoot blight emergence in Europe among which: (i) the replacement of the European native populations of *S. sapinea* by new populations or cryptic species of the pathogen, (ii) climate change that might influence both host susceptibility and pathogen presence or (iii) factors increasing the host susceptibility such as anthropogenic increase in nitrogen deposition.

A change in the pathogen population during the 20th century could have been unnoticed since *S. sapinea* is a species complex. Three morphotypes (A, B and C) were described on the basis of cultural characteristics, isozyme patterns and conidial morphology (Palmer *et al.*, 1987). Later, de Wet *et al.* (2003) showed that the B morphotype can be defined as a separate species, *Diplodia scrobiculata*, using multiple gene genealogies, while the A and C morphotype were merged in the *D. pinea* species. *D. scrobiculata* was shown to be less aggressive than *D. pinea* in inoculation trials (Palmer *et al.*, 1987, Bihon *et al.*, 2010). Hereafter, *S. sapinea* will refer to *S. sapinea sensu lato*. In France, all reports refer to *S. sapinea sensu lato*, but the two morphotypes A and B were reported on a *Cedrus deodara* in 1993 (Morelet & Chandelier, 1993). The relative frequency of the two species on pines thus remains unknown.

Environmental changes are also a likely hypothesis to explain the emergence of *Sphaeropsis* shoot blight as disease outbreaks usually depend on the occurrence of particular conditions such as hail, drought or heat-wave (van Staden *et al.*, 2004, Zwolinski *et al.*, 1990). The reported increase in both summer temperature and drought frequency associated with climate change would thus presumably exacerbate the disease. *Sphaeropsis* shoot blight could be favoured both via direct effects on the pathogen and indirect effects via host susceptibility. Indeed, *S. sapinea* is a thermophilic species with an optimum temperature for growth near 30°C (Keen & Smits 1989, Desprez-Loustau *et al.*, 2007) and its growth rate should be favoured by climate warming. Indirect effects are also likely to be important as susceptibility of pines to *D. pinea* is strongly enhanced by water stress (Blodgett *et al.*, 1997). Altered nitrogen nutrition can also promote the disease and *Sphaeropsis* shoot blight outbreaks in the Netherlands were related to anthropogenic nitrogen deposition (van Dijk *et al.*, 1992; Stanosz *et al.*, 2004). *Sphaeropsis* shoot blight is a good model to sort out direct effects of environmental changes on the pathogen from indirect effects on the host

susceptibility as high levels of *Sphaeropsis* inoculum in the stands are not conditioned by a high disease severity but rather depend on the presence of infected cones. Indeed, *S. sapinea* readily colonizes pine cones and colonized cones are important sources of inoculum. Palmer *et al.* (1988) showed that abundance of inoculum from cones was of primary importance for disease development in red pines. However, *S. sapinea* can be heavily present even in stands not severely affected by *Sphaeropsis* shoot blight when conditions are not conducive to the disease (Palmer *et al.*, 1988, Fecci *et al.*, 2002, Munck & Stanosz, 2010).

Our study was focused on possible qualitative and quantitative changes in the pathogen that might explain the emergence of *S. sapinea* disease in pines stands in France. We specifically addressed the following points: (i) determine the relative frequency of the two *Diplodia* species in a systematic survey of the presence of the fungi on pine cones, (ii) determine whether a relationship between the France-wide spatial pattern in *S. sapinea* inoculum availability on cones and disease regional frequency can be found; regional *Sphaeropsis* shoot blight frequency was estimated using data from the DSF, the French forest health survey agency, (iii) test whether the recent change in climate could have contributed to the emergence of the disease in France by favouring *S. sapinea* presence in the stands through direct effects on the pathogen. For that, we fitted a model linking climate variables to the frequency of the pathogen on pine cones and studied the response of past and predicted future changes of the climate on the pathogen abundance in the stands.

Materials and methods

Plot selection and survey procedure for study of *S. sapinea* presence in pines stands

Pines plots were surveyed for presence of *S. sapinea* by rating the frequency of the pathogens pycnidia on cones collected on the ground. The frequency of *S. sapinea* on cones was taken as a measure of inoculum presence in the stands. Plots were selected among the 509 plots of the French part of the level 1 ICP European network for monitoring of forest damage (permanent plots located on a 16 x 16 km grid covering all of Europe). The stands are located with their GPS coordinates; position on a 1:25000 map and an orthophotography are available. In each plot, 20 contiguous trees, selected in the dominant strata were marked, determined to the species and have been surveyed annually since 1989 for crown condition. The sample is open, trees disappearing each year being replaced. We selected all the plots within this network which had at least four *Pinus nigra*, *P. sylvestris* and/or *P. pinaster* and trees over 20 year-old on which cone could be found (97 plots). The 51 plots with *P. nigra* and/or *P. sylvestris* were surveyed from June to November 2005 (12 *P. nigra* plots, 34 *P. sylvestris* plots, 1 plot with both *P. pinaster* and *P. sylvestris* and 2 with both *P. nigra* and *P. sylvestris*, see Fig. 1a). The 29 plots with *P. pinaster* were visited in January 2006. We also included in the survey four plots of *P. radiata* that did not belong to the level 1 ICP network and that were visited by the staff of the Département de la Santé des Forêts, one in Corsica, one in the Pyrenean piedmont and two in Brittany. They were treated as the other pine stands. Altogether, 194 *P. nigra*, 626 *P. sylvestris*, 40 *P. radiata* and 562 *P. pinaster* were rated.

In each selected plot, we sampled 10 cones on the soil at the base of 10 different pine trees in order to study the frequency of cone colonization by *S. sapinea* as a measure of the level of pathogen presence in the stand. The trees of the permanent plots (*P. nigra*, *P. sylvestris*, *P. radiata* and/or *P. pinaster*) were sampled in priority and additional neighbouring pines were sampled when needed. The sampled cones had been on the soil for about 1-3 years. Depending on the pine species, cones remain on the tree for 2 to 3 years before being shed. Thus the cone colonisation by *S. sapinea* was assumed to represent the average recent inoculum availability in the stand. After collection, the cones were put in paper bags and brought back to the laboratory for analysis.

Fungal isolations and species determinations

Samples were air-dried in the laboratory at room temperature and then stored at 4°C until processed. *S. sapinea* produces black pycnidia on the top of the cone scales (Fecci *et al.*, 2002). The presence of pycnidia was recorded for each cone by macroscopic examination. When pycnidia were present, conidia were examined to determine whether they corresponded to *S. sapinea* spores i.e. thick walled spores, hyaline to brown when mature, with 0-1 septum.

Four cone scales with pycnidia were randomly chosen from each cone for a fungus isolation procedure adapted from Vujanovic *et al.* (2000). Each scale was surface sterilized i.e. placed in 70% ethyl alcohol for 10 s, rinsed in sterile distilled water, put in 2.5% sodium hypochlorite for 1.5 min, rinsed twice for 5 min in sterile distilled water and finally dried on sterile filter papers. Then, half of the scales was placed on Malt Agar (MA, Difco) and the other half on Potato Dextrose Agar (PDA, Difco). The plates were incubated at 25°C in the dark. Putative *S. sapinea* isolates, i.e. fast growing cultures visible after 4 days with aerial mycelium and grey to black colour, were then isolated in pure culture and transferred to PDA Petri dishes containing a sterilized pine needle and incubated at 25°C for 7 days in the dark and then about 10 days in the light. Cultures were determined to be *S. sapinea* when pycnidia with typical brown spores were produced on the needle.

A sub-sample of the *S. sapinea sensu lato* isolates was determined for species, i.e. *Diplodia pinea* or *D. scrobiculata*, by a species-specific polymerase chain reaction (PCR) test developed by Smith and Stanosz (2006). At least one isolate per cone was selected. If, based on morphology of the culture, isolates suspected of belonging to different *Diplodia* species were present on a cone, all the available isolates from the cone were determined for species. Blocks of agar were cut from culture collections and transferred to PDA Petri plates. Cultures were incubated at 25°C for 5 days. A rapid DNA extraction was done: non pigmented fungal mycelium from the active culture margin was scraped and put in 100 µl of 10x Tris-EDTA buffer and was then incubated for 5 min at 98°C and put in ice for 3 min; this sequence was repeated one time. The PCR reactions were performed as described by Smith and Stanosz (2006). All samples were tested for the presence of both *D. pinea* and *D. scrobiculata* using the respective specific primers DpF/BotR and DsF/BotR (Smith & Stanosz, 2006). Negative and positive controls were always included i.e. no DNA, DNA from CMW190 for *D. pinea* and CMW4898 for *D. scrobiculata* (de Wet *et al.*, 2003). The amplification reactions were carried out on a Genamp 9700 thermocycler (Applied Biosystems, Foster City, California).

Model of *S. sapinea* prevalence on pine cones

The prevalence of *S. sapinea* on cones, i.e. proportion of cones infected by the pathogen, was analyzed by logistic regression, using the GENMOD procedure of SAS/STAT 8.1 (SAS Institute, Cary, NC). This type of analysis assumes that the response variable follows a binomial distribution and that a function of the response (the link function) is linearly related to the independent variables (Collet, 1991). We used the Logit function as link function. Models were selected by a forward selection procedure, choosing at each step the variable that induced the largest drop in deviance.

Independent variables that might explain the frequency of *S. sapinea* on cones were stand characteristics such as pine species, age, stand origin (natural or planted), altitude and climate variables. The stands characteristics were provided by the Département de la Santé des Forêts (Nancy, France). Most natural stands were *P. sylvestris* in the mountain regions of Alps and Massif Central (20 / 31). However a limited number of natural stands occurred in plain areas (seven *P. sylvestris*, two *P. nigra* and two *P. pinaster* stands). Climate variables for each plot were taken from the nearest meteorological station of the Météo-France network with available data over the 1990-2005 period for monthly rain and average temperature (1244 stations in total). Average climate across a long 15 years period was

chosen as inoculum on cones was assumed to better reflect long term *Sphaeropsis* presence in the stand. The median distance between the plot and the meteorological station was 9.9 km (range 1-20 km). The climatic variables tested were the means over the 1990-2005 period of minimum, maximum and mean daily temperatures, sum of precipitations (in mm) and water balance (precipitation minus evapotranspiration, in mm) for four periods, i.e. winter (December to February), spring (March to May), summer (June to August) and autumn (September to November).

Estimation of the regional frequency of *Sphaeropsis* shoot blight

The database of the Département de la Santé des Forêts (DSF) stores records of health problems detected in France by a network of foresters trained to diagnose all types of forest health affections, including abiotic, entomological or pathological damages. DSF observer report health problem that they judge significant, i.e. that disturb stand management or that is of unusual severity for the area. Altogether, from 1989 to 2006, about 12800 records of health problems on *Pinus* species were entered in the database, 1064 of which concerned *Sphaeropsis* shoot blight. This database was used as an estimate of the regional frequency of *Sphaeropsis* shoot blight in pine stands. The non-systematic and non-homogeneous way used for data collection and the fact that only occurrence is recorded cause difficulties for the analysis of those data and does not allow the estimate of frequency of infected host. In particular, in order to interpret a local number of disease records, there is a need to estimate a local expected number of records that strongly depend on the local density of host and on the activity of the local people who produce the records. The methodology used was adapted from what is done in medical epidemiology (Lawson, 2001). Basically, the raw number of records of the studied disease is compared to the raw number of records for a set of problems selected to be typical of the host population of interest (the so-called 'at risk' population). The local density of reference records is considered to be an estimate of the at-risk population local surface and of the observation pressure by local observers. Reports on the 3 main hosts were used, *P. nigra*, *P. pinaster* and *P. sylvestris*. *P. halepensis* was not used because few *S. sapinea* reports have been made on that host (about 10). *P. pinea* and *P. uncinata* reports were not used although *S. sapinea* reports are significant on those hosts because those species are not well enough represented in the DSF database. We used as reference reports all non *S. sapinea* reports except reports on *Thaumetopoea pityocampa* and *D. septospora* that were very frequent and showed a strong spatial pattern. Altogether, 1019 *S. sapinea* reports and 7646 reference reports were used (Fig 2a).

All records from 1989 to 2006 were pooled to estimate average local disease severities. Local density of *Sphaeropsis* shoot blight and reference records were computed using nonparametric kernel estimation method (Wand & Jones 1995). This method estimates the record density on each point of a grid. Each record is weighed by the kernel function according to the distance to the grid point. The bandwidth is the important kernel parameter controlling the weight decay with the distance to the grid point. We computed the records density for each point of a 10 x 10 km grid. The quartic kernel with a bandwidth of 80 km was used for both *Sphaeropsis* shoot blight and reference records density. The least square cross-validation (Wand & Jones, 1995) was used to estimate the values of the bandwidth on several portions of the surveyed area, and to choose one which was large enough to avoid cases lacking record within the discus defined by the bandwidth.

A standardized record rate (SRR) was produced by dividing the *Sphaeropsis* shoot blight records density by the reference record density. Therefore, an estimate of SRR for the 1989-96 period was computed for each point of the 10 x 10 km grid. However, the report rate of *Sphaeropsis* shoot blight is very different on its different hosts (*P. nigra*, *P. pinaster* and *P. sylvestris*) and this had to be taken into account to avoid that the map merely shows the distribution of the most susceptible host. Therefore, records density of both *Sphaeropsis*

shoot blight and the reference were computed for each host (*P. nigra*, *P. pinaster* and *P. sylvestris*) and the SRR for the disease was computed as:

$$\text{SRR} = \frac{\sum_{\text{host}} (\text{SSB_report}_{\text{host}})}{\sum_{\text{host}} (\text{Reference_report}_{\text{host}} \times \text{mean_report_rate}_{\text{host}})}$$

With $\text{SSB_report}_{\text{host}}$ and $\text{Reference_report}_{\text{host}}$, the density of *Sphaeropsis* shoot blight or reference report per host, $\text{mean_report_rate}_{\text{host}}$, the ratio between the total number of *Sphaeropsis* shoot blight reports and the total number of reference reports for a particular host. All these analyses were done using the R statistics software (R Foundation for Statistical Computing).

Comparison of estimated past, present and future prevalence of *S. sapinea* on cones

The fitted model developed to explain prevalence of *S. sapinea* on cones was used to evaluate the potential impact of past and future climate on presence of *S. sapinea* inoculum in the stands. For past climate, we used the AURELHY data that are spatialized means over the 1961-1990 period for the monthly average temperature and rain provided by Météo-France. These spatialized data are computed for each point of a 1 x 1 km grid over France by a method taking into account topography. For future data, we used data from ARPEGE, a global circulation model (Déqué *et al.*, 1994). The model calculates relevant meteorological parameters on a grid of 360 points in France for the period 1960 to 2100 according to several scenarios of the evolution of atmosphere composition. The calculation of radiation includes the effect of four greenhouse gases (CO₂, CH₄, N₂O and CFC) in addition to water vapour and ozone, and of five aerosol types (land, sea, urban, desert and sulphate) in addition to background aerosols. The model used was forced by an effective greenhouse effect corresponding to the provisional IPCC2001 B2 scenario (Garrett *et al.*, 2006; Desprez-Loustau *et al.*, 2007). This CO₂ emission scenario is mild; it was however kept to enable comparison with others projections made on the impact of climate change on French forest ecosystems.

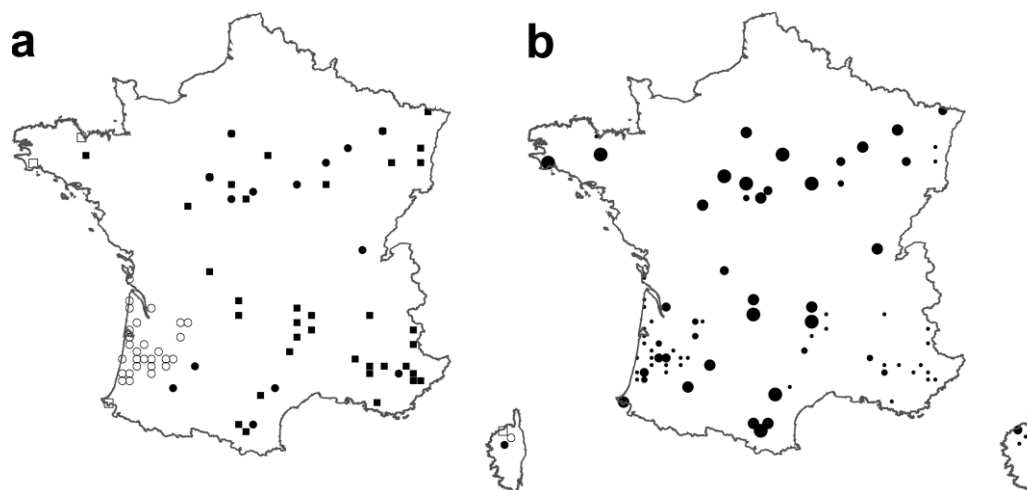


Fig. 1. Plots visited during the survey. A, pine species presence on plots, ●, *P. nigra*, ○, *P. pinaster*, □, *P. radiata*, ■, *P. sylvestris*. B, prevalence of *Diplodia pinea* on cones, ◦, 0%, ◐, 33%, ◑, 66%, ◒, 100%.

To study whether the past and future evolution of climate could favour presence of *S. sapinea* in pine stands, maps of the pathogen prevalence on cones of *P. nigra* / *P. sylvestris* for the 1961-1990, 1991-2005 and 2030-2060 periods were derived from the previously developed logistic model fed with the various climatic datasets: the Aurelhy data (1961-90), the 1991-05 meteorological data used to initially develop the model and the ARPEGE data

for the 2030-60 periods, adapting the anomaly method used by Bergot *et al.* (2004). Briefly, evolutions of the relevant meteorological parameters from the 1961-90 to the 1991-2005 period were computed for each of the available meteorological stations. Only the 109 meteorological stations with data available from 1961 to 2005 were used. Relevant meteorological parameters for the Aurelhy grid were computed by summing the 1961-90 Aurelhy data and the 1960-90 to 1990-2005 evolution at the nearest available meteorological station. The same technique was used to produce the 2030-60 map of *S. sapinea* prevalence on cones. The potential evolution in predicted *S. sapinea* prevalence on pine cones at the 109 meteorological stations with 1960-2005 data and at the 360 ARPEGE grid points with 1990-2060 data was tested by paired *t*-test.

Results

S. sapinea prevalence on cones

Strong heterogeneity was found in the colonization of cones by *S. sapinea*, with prevalence ranging from 0 to 100% of cones colonized per stand (Fig. 1b). The pathogen was detected on all studied pine species in all the country. A total of 300 fungal isolates were analysed for the species determination. The species found to be present most of the time was *Diplodia pinea*. *D. scrobiculata* was observed in only three cones in a single *P. radiata* stand in Corsica and these cones were also colonized by *D. pinea*. Thus, the study hereafter only refers to *D. pinea*.

Table 1. Odds ratio associated with the fitted multivariate model explaining prevalence of *Diplodia pinea* on pine cones in France.

Factor	Odds Ratio (Confidence limits)	P value
Host species: <i>P. sylvestris</i> versus <i>P. pinaster</i>	10.9 (4.9 – 24.6)	< 0.001
<i>P. nigra</i> versus <i>P. pinaster</i>	12.1 (5.6 – 26.1)	< 0.001
<i>P. radiata</i> versus <i>P. pinaster</i>	NS	0.397
Mean minimum daily temperature in winter (per 1°C)	1.6 (1.4 - 1.8)	< 0.001
Rain in summer (per 10 mm)	1.07 (1.02 - 1.13)	0.005

NOTE: Model was fitted with scale factors of deviance / df value, the deviance / df of 2.0 indicating overdispersed data.

Three main factors explained the heterogeneity of cone colonization. Strong differences were found in the colonization of cones between the different pine species (Table 1, Fig. 3). *P. nigra* and *P. sylvestris* cones were colonized 11-12 fold more often than *P. pinaster* cones (Table 1). No significant difference in colonization between *P. nigra* and *P. sylvestris* cones was detected ($\chi^2 = 0.42$, $P = 0.520$). High winter temperature had a positive impact on the frequency of cone colonization by *D. pinea*: for each additional 1°C on the mean winter daily minimal temperature, cone colonization was multiplied by 1.6 (Table 1, Fig. 3b). This probably explains that cone colonization was inversely proportional to altitude (Fig. 3a), with a significant relationship ($\chi^2 = 16.81$, $P = 0.001$). Finally, rain in summer increased slightly but significantly the pathogen prevalence on cones (x 1.07 for each additional 10 mm of summer rain, Table 1).

By contrast, several factors were not linked with cone colonization by *D. pinea*, in particular pine age ($\chi^2 = 0.05$, $P = 0.819$). Stand origin, i.e. natural or artificial, was significantly linked to the level of pathogen presence on cones ($\chi^2 = 38.01$, $P = 0.001$).

However, this was a spurious correlation linked to the fact that most natural stands were located at high elevation, in areas with cold winters and had cones poorly colonised. When introduced in a model already including winter mean of minimum daily temperatures, the stand origin was not significantly linked to the prevalence of *D. pinea* on cones ($\chi^2 = 0.13$, $P = 0.720$). Indeed, we noticed that cones in stands of natural origin in plains were usually heavily colonized.

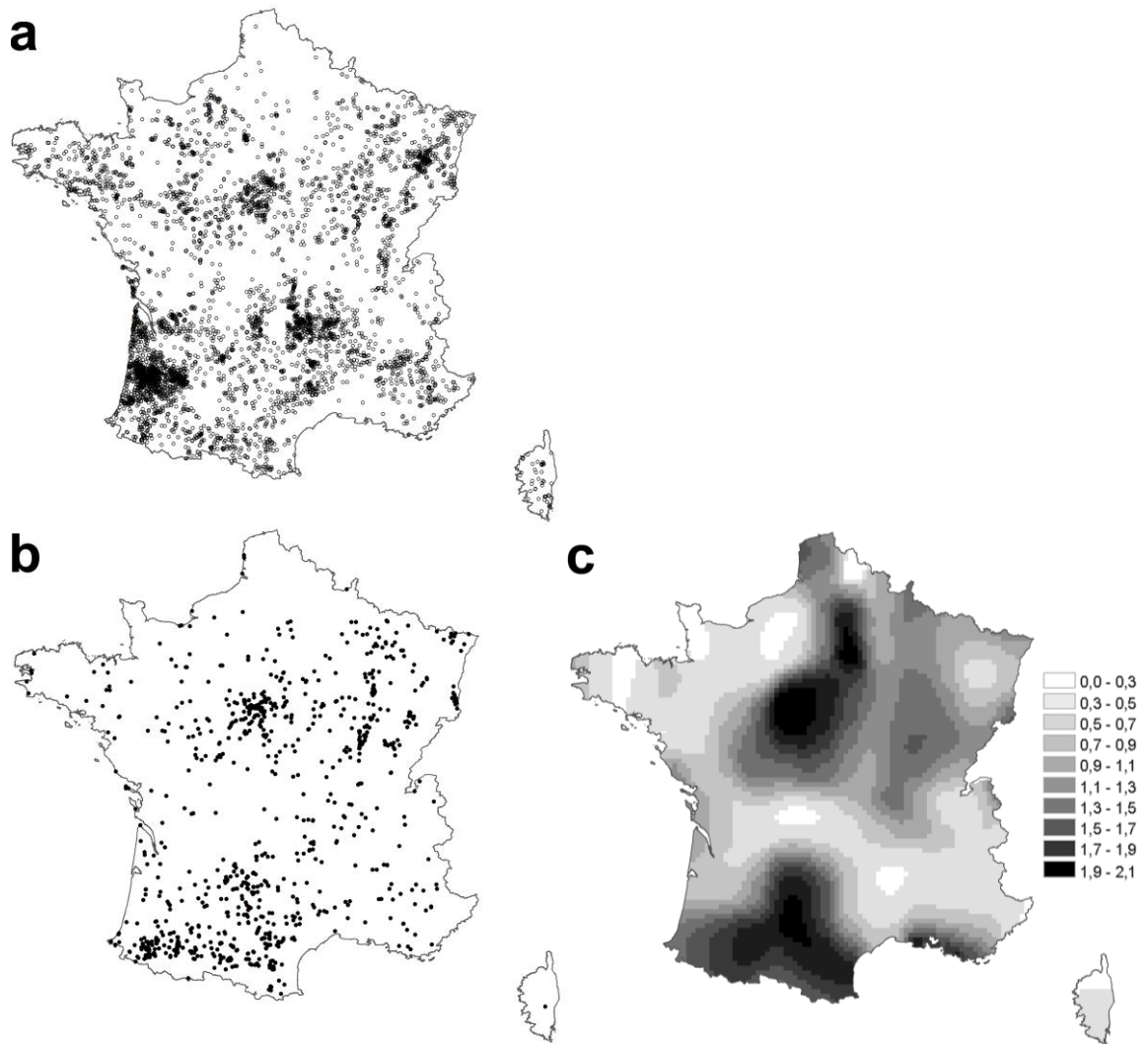


Fig. 2. Report of *Sphaeropsis* shoot blight in the DSF database (1989-06). a. reference report (○). b. *Sphaeropsis* shoot blight reports (●). c. standardised report rate.

Comparison between pattern of prevalence on cone and *Sphaeropsis* shoot blight report

Figure 2b shows the spatial pattern of *Sphaeropsis* shoot blight report in the DSF database from 1989 to 2006. The report is especially high in SW and central France with SRR over one, while SRR in NW and mountain areas such the Alps and Massif Central are lower than one indicating *Sphaeropsis* shoot blight reports less frequent than average. This spatial pattern in SRR shows similarity with the spatial pattern in *D. pinea* prevalence on

cones (compare Fig. 1b and Fig. 2b). The relationship is highly significant when comparing the *D. pinea* prevalence on cones measured in each surveyed plot with the estimated SRR at the location ($F = 27.10$, p value < 0.0001). Figure 4 shows that the *Sphaeropsis* shoot blight SRR increases with the observed *D. pinea* prevalence on cones.

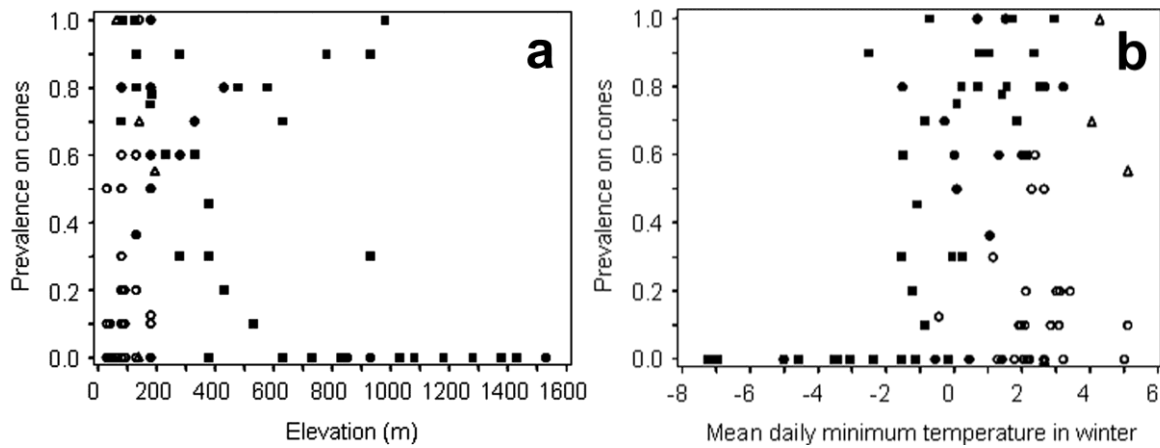


Fig. 3. Influence of elevation and temperature on cone colonization by *Diplodia pinea*. a. Elevation ($\chi^2 = 16.81$, $P = 0.001$); b. mean daily minimum temperature in winter (December to February, $\chi^2 = 31.05$, $P = 0.001$). •, *P. nigra*, ○, *P. pinaster*, △, *P. radiata*, ■, *P. sylvestris*.

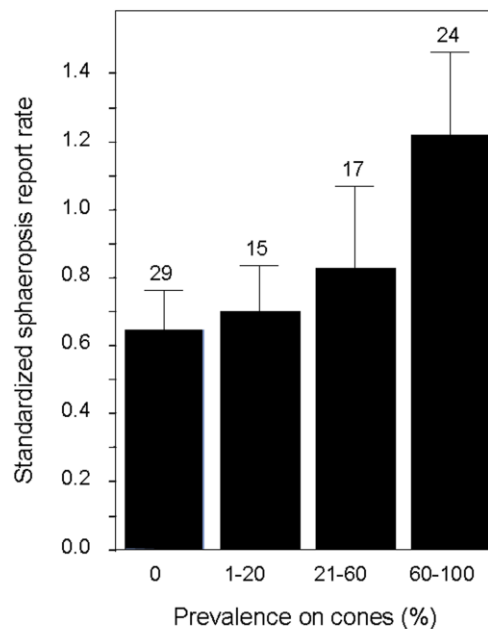


Fig. 4. Relationship between regional severity of *Sphaeropsis* shoot blight and prevalence of *D. pinea* on cones. The bars represent the confidence interval for the mean.

Predicted evolution of *D. pinea* prevalence on cone with climate change

We produced maps representing predicted colonization of *P. nigra* and *P. sylvestris* cones by *D. pinea* in France according to the model presented in Table 2, i.e. with winter temperature and summer rain, for three periods, 1961 to 1990, 1990 to 2005 and 2030 to 2060. Compared to data used for model development, we extrapolated to dryer and hotter situations. However, the areas concern by this extrapolation are very limited Mediterranean and Atlantic coastal regions (3.5% of the area for the 2030-60 period) and this has very little impact on the global prediction. The predicted prevalence of *D. pinea* on cones significantly increased from 1960-90 to 1990-2005. Indeed, the mean predicted prevalence at the 109 studied meteorological stations was multiplied by 1.14 between the two periods (paired *t*-test = 14.55, $P < 0.001$). The increase was mainly explained by an increase in the daily minimum temperature in winter (+ 0.7°C) while the summer rain increased slightly (+ 3.7 mm). The comparison of maps from Figures 5a and 5b shows an eastward expansion of areas favourable to the presence of *D. pinea* in the pine stands, while the situation has not changed in mountain areas that remain unfavourable or on the Atlantic shore that remains favourable. When the predicted prevalences for the periods 1990 to 2005 and 2030 to 2060 were compared, the increase was also significant, but slighter, with prevalence multiplied by 1.05 (paired *t*-test = 16.86, $P < 0.001$). The impact on *D. pinea* prevalence on cones of the predicted increase in winter daily minimum temperature from 1990-2005 to 2030-60 (+ 0.7°C) was less significant because the 1990-2005 winter temperature levels are already favourable to *D. pinea* in most French low lands; the predicted increase in *D. pinea* prevalence on cones of the increase was also slightly mitigated by a predicted decrease in summer rain (- 23.0 mm).

Discussion

We found that the most frequent *Diplodia* species present in pine stands in France is *D. pinea*. The data are consistent with the hypothesis that recent climate warming might have contributed to the emergence of *Sphaeropsis* shoot blight in France by a direct positive impact on the pathogen. Indeed, spatial pattern of *D. pinea* frequency in stands and *Sphaeropsis* shoot blight report by the forest health survey system (DSF) were correlated. Frequency of *D. pinea* in pines stands was mainly explained by the host species and by climate, especially high winter temperature. The climate became more favourable to *D. pinea* presence in pines stands within the last 15 years compared to the previous 30 years period. By contrast, future climatic changes over the next 40 years as predicted by Météo-France should have far less impact on the pathogen frequency on cones.

Diplodia pinea was the only species found on the cones of *Pinus pinaster*, *P. sylvestris*, and *P. nigra* collected on the soil. Thus, French pine stands are exposed to the aggressive *D. pinea* species which has often been associated with induction of twig necrosis while *D. scrobiculata* is less aggressive (Palmer *et al.*, 1987, Bihon *et al.*, 2010). The distribution of *D. scrobiculata* within France, only found on *P. radiata* in Corsica, is interesting. Indeed, the pathogen has yet been reported in Europe sporadically in mediteranean areas (Morelet & Chandelier, 1993), in South Africa (Bihon *et al.*, 2010) and is the only species found on the native *P. radiata* in California (Burgess *et al.*, 2001). However, the limited distribution of *D. scrobiculata* in France cannot be explained only by host preference as, although only *D. pinea* is reported from *P. pinaster* and *P. nigra*, both *D. pinea* and *D. scrobiculata* have been observed on *P. sylvestris* and *P. radiata* (Stanosz *et al.*, 1999; de Wet *et al.*, 2003). In view of the very limited distribution of *D. scrobiculata* in France, the hypothesis that the replacement of this species by the more aggressive *D. pinea* could have caused the emergence of the *Diplodia* shoot blight in France in the early nineties is not likely. Indeed, although the two pathogens were always found competing on the same cones, the complete elimination of *D. scrobiculata* from most of French pine stands through competition with *D. pinea* would be surprising as the two pathogens coexists in pines stands of the central part of USA (Palmer *et al.*, 1987). However, we cannot rule out this hypothesis.

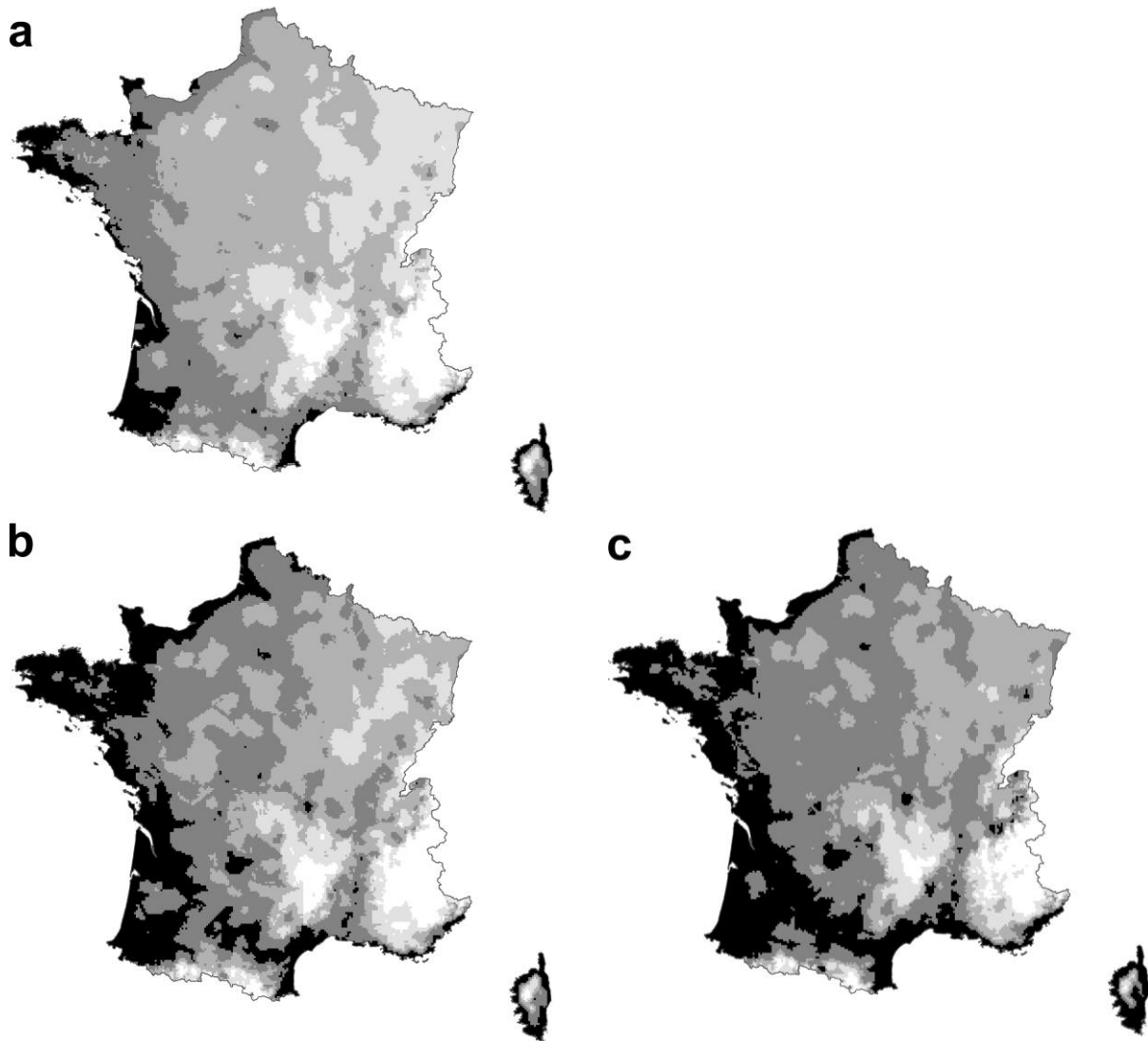


Fig. 5. Maps of cone colonization in France by *Diplodia pinea* for *P. nigra* and *P. sylvestris* predicted by the fitted model for periods: a. 1960-1990, b. 1990 to 2005, c. 2030 to 2060. The fitted model is: $\text{Logit}(p) = -1.83 + 0.007 \times \text{Rain}_{\text{sum}} + 0.45 \times \text{Tn}_{\text{win}}$, with p , the proportion of colonized pine cones, Rain_{sum} the annual rain in June-August and Tn_{win} the mean daily minimum temperature in December-February. □, 0-20%, ◻, 20-40%, ◼, 40-60%, ◽, 60-80%, ◼, 80-100%.

We documented a positive relationship between regional *Sphaeropsis* shoot blight report by the DSF and *D. pinea* frequency on cones in studied stands. This result is not easy to interpret in terms of causal relationships. On the one hand, it has been reported that severe *Sphaeropsis* shoot blight is conditioned by high inoculum level from cones (Palmer *et al.* 1988), but on the other hand, an increase in inoculum in areas favourable to disease expression cannot be ruled out. However, our data show that significant disease levels are not a prerequisite for high colonisation rate of cones: a high frequency of *D. pinea* on cones together with low local report of *Sphaeropsis* shoot blight was observed in some areas (Massif Central, Brittany in the NW). Indeed, climatic conditions favourable for cones colonisation and for *Sphaeropsis* shoot blight are different. If high temperatures are needed for both, strong cone colonisation by *D. pinea* was related to high summer rainfall will severe *Sphaeropsis* shoot blight is known to be associated with drought (Bachi & Peterson, 1985; Blodgett *et al.*, 1997). The importance of drought for disease occurrence explains the

variability in regional *Sphaeropsis* shoot blight standardized report at a given level of cone colonization.

One of the main factors explaining the prevalence of *D. pinea* on the cones was the host species. Although *P. sylvestris* has been reported to be less susceptible to *D. pinea* than *P. nigra* (Vujanovic *et al.*, 2000), this was not paralleled by a higher presence of the pathogen on the cones of this species. By contrast, *P. pinaster* cones were less frequently colonized by *D. pinea* than cones of other pines species. The low frequency of *D. pinea* in the Landes area, in south-western France on *P. pinaster* cones cannot be explained by an unfavourable climate as the area is submitted to mild winter temperature and high rainfall. This low inoculum level in the *P. pinaster* stands of SW France parallels low *Sphaeropsis* shoot blight report in the area. This cannot be explained by resistance of *P. pinaster* to *Sphaeropsis* shoot blight as this species showed moderate to high susceptibility to *D. pinea* when tested in artificial inoculation (Swart *et al.*, 1988). Alternative hypotheses are that either *D. pinea* has a poor ability to colonise *P. pinaster* cones or that the area has not yet been colonised by the pathogen for historical reason. A possibility could be that until the last decades, *P. pinaster* stands in the Landes area were installed by sowing and not by planting nursery seedling that are known to be possible sources of *D. pinea* inoculum (Palmer *et al.*, 1988).

Both winter temperature and summer rainfall explained the local prevalence of *D. pinea* on pines cones. The positive effect of winter temperature can be explained by the high thermal requirements of the fungus (about 30°C for optimal growth, Keen & Smits 1989, 8°C as a minimal threshold for growth, Desprez-Loustau *et al.* 2007). The low prevalence on cones in areas of low winter temperature probably explains the decreased presence of the pathogen at high elevation, which has already been mentioned in the literature (Zwolinski *et al.*, 1990). The low impact of *D. pinea* at high elevation might be caused by low presence of inoculum in the stands more than to conditions not conducive to disease expression. Indeed, the pine species present in the mountain areas, *P. nigra* and *P. sylvestris* are susceptible hosts the cones of which are usually well colonized. High rain in summer has already been mentioned in literature to be favourable to development of *D. pinea*. Indeed, Peterson (1977) showed that the pathogen extensively develops pycnidia on second-year seed cones and needles in the autumn if late summer rain is abundant.

The fitted model linking the pathogen presence in the stands to climate allowed us to produce a map of the French current situation but also to simulate maps of the past and future situations. Clearly, climate became more favourable to the pathogen presence in the stands in the last 15 years compared to the 1960-90 period. This evolution of the climate could at least partly explain the emergence of the disease in the 90^{ies} in France. Indeed, especially in central France where the disease was severe during the last outbreaks (Piou *et al.*, 1991), the French climatic conditions were far less favourable to the pathogen development in the 1960-90 period than in the current period. By contrast, the simulated future evolution according to our model showed limited increase of *D. pinea* presence over France. In particular, mountain areas are predicted to remain unfavourable to the pathogen. This may be explained because most of climate constraints on pathogen presence were relieved in the recent decade in the lowland area of France and only the mountain areas remain unfavourable to the pathogen presence on cones. Future increase in temperature thus relieve constraints on pathogen presence in area of limited surface at the periphery of mountains. Also, it has to be pointed out that the B2 CO₂ emission scenario that we used is mild which could explain this moderate projected evolution. However, projections made on other tree diseases or on tree species distribution under the B2 emission scenario often showed large increases in the species presence (Bergot *et al.* 2004; Badeau *et al.*, 2010).

Few studies explore the impact of climate changing on plant diseases and even fewer demonstrated a causal relationship between climate change and the recent emergence of plant diseases (Garrett *et al.*, 2006) although some cases were documented for forest diseases (Wood *et al.*, 2005). Most of the studies only consider the impact of increasing

temperature, improving either overwintering or pathogen population increase during the vegetation season. This approach was used to model the epidemics of oak disease caused by *Phytophthora cinnamomi* in Europe under climate change (Bergot *et al.*, 2004; Brasier *et al.*, 1994). It was shown that climate warming could allow the disease expansion of one to a few hundred kilometres eastward from the Atlantic coast within one century. The originality of our study is that both changes in temperature and precipitation were taken into account in the model. Host-pathogen interactions are complex and climatological factors vary simultaneously in a dynamic environment. Few studies have been done to model effects of climate change with several environmental factors. For example, Evans *et al.* (2008) developed models based on temperature, precipitation and elevated CO₂ and proved the increasing potential for phoma stem canker, caused by *Leptosphaeria maculans* under global warming. Desprez-Loustau *et al.* (2007) showed that for foliar forest diseases such as Dothistroma needle blight, the increase in temperature could be balanced by a reduction in precipitation mitigating the impact of climate change on the disease. The model developed in this work takes into account only direct impact of climate change on pathogen presence in stands. However, there is a need for more complete models taking into account the impact of water shortage on the host tree physiology. Indeed, although climate evolution might not be more favourable to pathogen presence on cones, the predicted increases in the frequency and severity of drought should favour *Sphaeropsis* shoot blight outbreaks.

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