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### ► To cite this version:

Marie Grosdidier, R. Ioos, Benoit Marçais. Do higher summer temperatures restrict the dissemination of *Hymenoscyphus fraxineus* in France?. *Forest Pathology*, 2018, 48 (4), pp.e12426. 10.1111/efp.12426 . hal-01893934

**HAL Id: hal-01893934**

**<https://hal.science/hal-01893934>**

Submitted on 30 Jan 2020

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## Do higher summer temperatures restrict the dissemination of *Hymenoscyphus fraxineus* in France?

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### Abstract

*Hymenoscyphus fraxineus* is an invasive pathogen that severely affects European ashes, jeopardizing the use of this species in forestry. However, even aggressive invasive pathogens require environmental conditions conducive to disease development. Hence, *H. fraxineus* survival at temperatures above 35°C is limited, which could reduce its impact in southern Europe. This study examined the effect of environmental conditions, mainly summer temperatures, on ash dieback development in southeast France between 2015 and 2016. Fourteen sites were selected according to altitudinal and longitudinal gradients covering a wide range of mean summer temperatures. Disease severity, density of infected and healthy rachises in the litter, and quantity of apothecia produced on infected rachises were measured. Results point to limited disease severity in this area because of the occurrence of high summer temperatures that are unfavorable to the pathogen. In a context of global warming, increases in temperatures could have a positive impact on ashes by limiting ash dieback development.

### Introduction

Epidemiologists often describe disease with the so-called disease triangle, where disease occurrence derives from the encounter of an aggressive pathogen with a susceptible host, in a conducive environment. Invasive diseases may have dramatic consequences because the pathogen and the host have never co-evolved and the host population may have very low resistance. Dutch elm disease represents an extreme example of this with very high mortality induced by *Ophiostoma novo-ulmi* in the European and North American elm populations after the disease emerged in the 1970's (Gibbs, 1978 ; Brasier and Buck, 2001). However, even with very aggressive invasive pathogens, a conducive environment is directly correlated with very severe disease impact. For example, environmental conditions were conducive to Dutch elm disease in large parts of the world, in particular in France, with daily mean air temperatures higher than 17°C during the months of June to August (Sutherland et al., 1997), enabling the disease to develop with little restriction. When environmental conditions are less favorable, the disease impact may be more limited. Elm trees growing in Scandinavia are less affected by Dutch elm disease because survival of the beetle vector is limited by harsh winters (Solheim et al., 2011). Another example is alder decline induced by *Phytophthora xalni*. The pathogen's limited survival at low winter

temperatures, as well as at high summer temperatures, strongly limits disease development in northeastern France or in southern Sweden for example (Aguayo *et al.*, 2014; Redondo *et al.*, 2015).

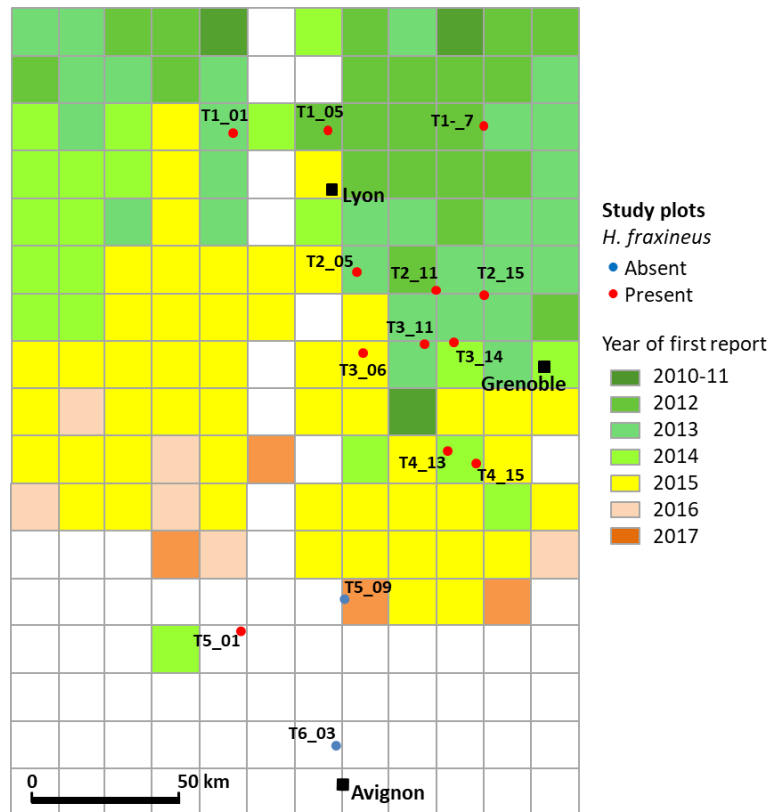
In the last two decades, a new ascomycete, *Hymenoscyphus fraxineus*, was introduced from Asia to Europe causing severe ash dieback. This fungus, described by Kowalski (2006), induces collar canker, shoot mortality and leaf necrosis, with severe economic, environmental and social consequences. In France, *H. fraxineus* was first observed in 2008 in Haute-Saône and has since spread toward the west and south of the country with a spread speed of about 50 km per year (Grosdidier, unpublished results). Ascospores released in the air infect ash leaves and, under adequate conditions, the pathogen may then transfer to the stems during the following autumn and winter, leading to dieback in the next spring. After leaf fall, the fungus overwinters on ash rachises in the litter where it forms a pseudo-sclerotial plate which protects it from desiccation. In spring, reproduction occurs if the two sexual types of the fungus mate on a rachis, leading to the production of apothecia and to the release of infectious ascospores. The disease is influenced by environmental conditions. High site humidity also enhances the severity of collar canker on the ash trees (Marçais *et al.*, 2016). Moreover, Kowalski & Bartnik (2010) have shown that the optimal *in vitro* growth temperature of the fungus is around 20°C. This favorable environment is found in most of Europe, which allowed *H. fraxineus* to spread rapidly with high impact across the continent. However, Kowalski & Bartnik (2010) and Hauptman *et al.* (2013) showed in laboratory experiments that temperatures above 30°C are lethal to the pathogen. Its mycelial development is stopped and the fungus can no longer be isolated from ash stems after exposure of a few hours to 35°C. This could limit disease development for instance in Slovenia and perhaps in other parts of southern Europe.

**Table 1:** Characteristics of the studied plots

Site	E	N	Flood risks index	Mean of maximum temperatures in			Evolution of dieback severity (2016-2015)	Nb hours T>35°C (2015)
				2014	2015	2016		
T1_01	45.93383	4.42987	0	21.86	25.79	22.80	-1.79	2
T1_05	45.93368	4.84273	0	22.83	26.71	24.25	0.68	1
T1_17	45.92506	5.51949	0	20.46	24.11	22.45	8.27	0
T2_05	45.50435	4.94965	1	23.36	26.82	24.86	4.41	0
T2_11	45.43761	5.28539	0	21.88	25.39	23.72	3.87	0
T2_15	45.41548	5.49142	0	21.18	24.57	22.73	0.42	0
T3_06	45.25595	4.95314	1	23.84	27.18	25.42	-1.37	1
T3_11	45.27661	5.21571	0	20.84	24.14	22.11	10.50	0
T3_14	45.27325	5.34408	0	21.00	24.29	22.22	-0.48	0
T4_13	44.94336	5.30884	0	19.16	22.92	20.95	7.81	0
T4_15	44.90220	5.42639	1	18.02	21.37	18.96	14.11	0
T5_01	44.42482	4.40577	0	25.97	29.37	27.39	0.56	5
T5_09	44.50658	4.84779	1	25.06	28.27	26.22	-	2
T6_03	44.06285	4.79562	1	27.42	29.93	28.58	-	4

The objective of this study was to test the effect of high summer temperatures and site humidity, which are known to influence pathogen development in the laboratory. In the field, a significant effect of humidity on disease development has already been demonstrated but not of

high summer temperatures. For this purpose, we focused the study on southeast France where ash dieback has emerged since 2013 and where summer temperatures might reach levels unfavorable to *H. fraxineus*, taking advantage of a large temperature gradient in the area between the Rhône valley and the surrounding mountains.



**Fig. 1:** Maps of the 14 sites with reports of disease presence by the Forest Health Department since 2010. Squares of the figure represent year of the first disease reports in the area of the square, by the Forest Health Department.

## Material and Methods

The South of France has a Mediterranean climate with summer temperatures that frequently exceed 30°C. The Rhone Valley is an area which alternates mountainous areas with the Alps and Massif Central, and a central valley with the Rhône River. Differences in elevation allow the creation of temperature gradients oriented from West to East in addition to the North to South gradient. While ash dieback has been reported by the French Forest Health Department (DSF, *Département de la Santé des Forêts*) since 2012 in the north of the area, the southern part has remained disease-free until now. Fourteen sites were selected in six transects in the Rhône Valley (Figure 1 and Table 1). Sites were chosen according to altitudinal and longitudinal gradients to sample a wide range of mean summer temperatures. The selection of potential sites was first based on the presence of water courses and tree cover using Google Earth images, then on visits where only sites with the presence of at least 15 ashes on a 35 x 35 m plot (about 1250 m<sup>2</sup>) were selected. All sites included *F. excelsior* and *F. angustifolia*. More precisely, a majority of *F. angustifolia* was observed in four sites (i.e. T1\_01, T2\_05, T5\_09 and T6\_03), while *F. excelsior* was predominant in the others sites.

Between 15 and 63 ashes were marked per site and rated in early August of 2015 and 2016 for crown symptoms and collar canker, according to the following score: 0, absence of shoot mortality; 0.05, 1-10% shoot mortality; 0.3, 10-50% shoot mortality; 0.625, 50-75% shoot mortality; 0.875, 75-100% shoot mortality or trees with the main stem dead but presence of sprouts at the stem base; and 1, 100% shoot mortality (trees without any leaves but that were still standing). The global site decline index, hereafter called “ash dieback severity”, was computed as the average of tree crown symptom scores. The circumference of each ash trunk at 1m30 was measured. For each tree observed per site, we verified the presence of any collar rot by scraping the collar trees. A proportion of trees with collar canker per site was then computed.

In August 2016, the density of ash rachis in the litter was determined along a 10 meter transect placed under ash trees at the center of each site. All ash rachises observed in the litter within a 10 cm band along the transect were harvested. They were brought back to the laboratory where they were separated in two categories: infected by *H. fraxineus* (i.e. black or brown rachis with presence of a distinct pseudo-sclerotial plate) or healthy (i.e. white or beige rachis, without any pseudo-sclerotial plate). The density of rachises per site was computed as the total length of rachises divided by the surface of litter observed. The ratio between infected and healthy rachises was then computed. The number of apothecia observed on each infected rachis was then counted. No rachises and/or apothecia were harvested at site T5\_01, which was very difficult to access. The validity of the rachis infection rating was checked. To do this, 5 mm segments of 2 infected and 2 healthy rachises per site were sampled and added to 400 µL of AP1 lysis buffer, 4 µL of RNase and two tungsten beads in a 2 mL Eppendorf tube. Samples were ground twice using a Tissue Lyser (*Retsch, Haan, Germany*) set at 30 Hz for 45 sec. DNA extractions were conducted according to *Grosdidier et al.* (2017). Two negative controls (water) were included during the DNA extraction steps as quality controls. Real-time PCR (qPCR) amplifications were performed according to *loos et al.* (2009b). DNA extracts were analyzed by qPCR with the Brilliant II qPCR Master Mix (*Agilent Technologies, Santa Clara, CA, USA*) using a QuantStudio6 thermal cycler (*Life Technologies, Saint Aubin, France*). Two replicates were tested for each sample and for negative extraction controls. Four non-template controls (NTCs, i.e. water) were included in the run. Three DNA plasmids ( $2.4 \times 10^9$  pDNA copies) containing the *H. fraxineus* qPCR target sequence were included as positive controls of qPCR (*loos & Fourier, 2011*).

The year of disease arrival within the area was used as a surrogate for site invasion time and was retrieved from the French Forest Health Department (DSF, Figure 1), which has recorded ash dieback symptoms for the country since 2008. The number of years of disease presence was computed for each site as the difference between the first year of observations, i.e. 2015, and the first year the disease was recorded by the DSF in the quadrat containing the site. When disease had never been recorded in the quadrat, the time of presence was set at zero. Maximum and mean daily temperatures for 2014, 2015 and 2016 were retrieved for each site from the Safran database of Météo France; these data are meteorological data computed on a 16 x 16 km grid. The mean of the maximum daily temperatures in the summer period (June, July and August, MMDT) was then computed for each site for 2014, 2015 and 2016. For litter humidity, which may affect apothecia production, an index of the flood risk produced by the BRGM (French Geological Survey) ([www.inondationsnappes.fr](http://www.inondationsnappes.fr), date of data update 2011-12-15) was used as a surrogate. These flood risk indexes (FRIs) are available for grids of 100 x 100 m. A BRGM index of less than 5 was assumed to correspond to a significant flood risk, whereas a BRGM index of 5 and higher corresponded to a low flood risk.

Data were analyzed by “piecewise” Structural Equation Modelling (SEM) using the R package, which utilizes the Generalized Linear Model (GLM). The SEM model was built according to the following hypothesis. Dieback observed in a given year results from both the dieback level of previous years and the shoot infections experienced during the previous fall and winter, which

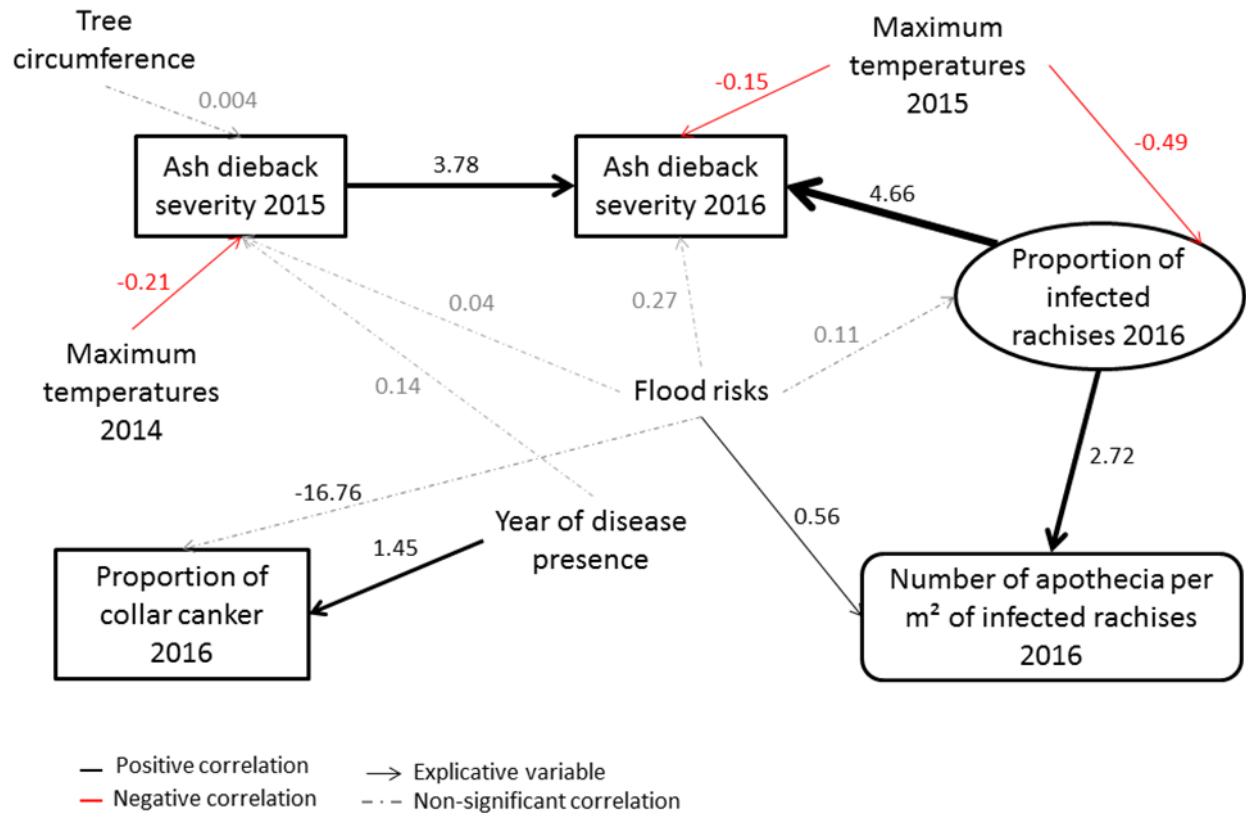
themselves depend on the foliar infection experienced during the previous summer. Thus, ash dieback severity observed in summer 2016 should be correlated to the severity in 2015, while severity in 2015 should be related to the number of years of disease presence. We took the proportion of infected rachises observed in the litter in 2016 as a proxy of foliar infection in 2015 and thus ash dieback in 2016 should be related to it. Last, environmental conditions such as temperature the previous summer and flood risks, used as a proxy of litter humidity, should influence the disease dynamics (foliar infection, dieback and basal canker prevalence), while tree size is known to influence dieback. The number of apothecia per length of infected rachis was assumed to depend on the density of infected rachis in the litter. We made this assumption because apothecia derive from the fertilization of infected rachis by conidia, acting as spermatia. This is especially the case at low infection levels when rachises should be infected only by one mating type (recently infected site or unfavorable conditions for leaf infection). A low density of infected rachis in the litter should limit the dispersal of conidia, which are supposed to be dispersed by rain splashing, presumably at short distances. Crown dieback and the proportion of rachises infected observed in the litter were assumed to follow a beta distribution and a logit link was used. These variables are proportions not based on a number of cases observed, which justifies the use of beta regression rather than binomial regression. The change in ash dieback was computed for each site as the 2016 minus the 2015 mean severity. When dieback change was negative, the site was classified as recovered, but when it was above zero, the site was classified as damaged. The change was tested with environmental variables in a Linear Model.

## Results

*Hymenoscyphus fraxineus* DNA was detected on 96% of the tested rachises classified as infected, i.e. in all sites tested except in two, T5\_09 and T6\_03, where no infected rachises were observed. All tested rachises classified as uninfected were negative for *H. fraxineus* DNA. The presence of *H. fraxineus* was confirmed by qPCR from an infected shoot sampled at site T5\_01, where no rachises were sampled. Fungus presence was thus confirmed by qPCR at all sites except T5\_09 and T6\_03, and these two sites were removed from further analysis.

Five sites presented high flood risks, while nine sites had low flood risks. The range of the mean maximum daily temperatures (MMDTs) recorded by Météo France in the sampling area between early June and late August was 18–27°C in 2014, 21–30°C in 2015, and 19–29°C in 2016. No hours with temperatures higher than 35°C were recorded at any sites during the summers of 2014 and 2016, but in summer 2015 up to 5 hours with temperatures higher than 35°C were recorded at some of the sites (Table 1).

Average ash dieback severity in 2015 across the 12 sites with presence of *H. fraxineus* was 23% (CI [14–31]). In 2016, ash dieback severity across the 12 sites was 25% (CI [16–33]). Collar cankers were present at only 4 sites, in the north of the area (T1\_01, T1\_05, T1\_17 and T3\_14), with an average proportion of 11% of the ashes with collar canker. Across the 12 sites, mean ash dieback change was 3.9 (CI 0.98–6.85) with a slight increase between 2015 and 2016. The change in ash dieback severity between 2015 and 2016 was negatively related to the 2015 mean maximum daily temperature (MMDT) recorded ( $p$ -value = 0.01), but not significantly related to the flood risk index (FRI) ( $p$ -value = 0.43).



**Fig. 2:** Structural Equation Modelling indicating all relationships between environmental influences and measured variables. The width of the line is proportional to the estimated coefficient of the parameter, which is indicated next to the line. Solid lines reflect significant correlations and dashed lines reflect non-significant correlations. Red lines reflect negative correlations and black lines reflect positive correlations.

Structural Equation Modelling (SEM) was performed and Figure 2 shows the relationships between each variable. The number of apothecia observed per meter of infected rachis was related to the proportion of infected rachises in the litter and environmental conditions at the site, with a GLM showing a distribution of the Poisson family, using the log of length of infected rachis as an offset. The proportion of collar canker observed at a site was related to the number of years with disease presence and environmental conditions, with a GLM showing a distribution of the binomial family. Tree sizes, as measured by the mean site diameter at breast height (DBH), were very similar among the sites and showed no significant relationship with ash dieback severity ( $p$ -value = 0.33). Ash dieback severity in 2015 was explained negatively by the 2014 MMDT ( $p$ -value = 0.04), but not by flood risk indexes (FRIs) or by the number of years with disease presence ( $p$ -values = 0.94 and 0.49, respectively).



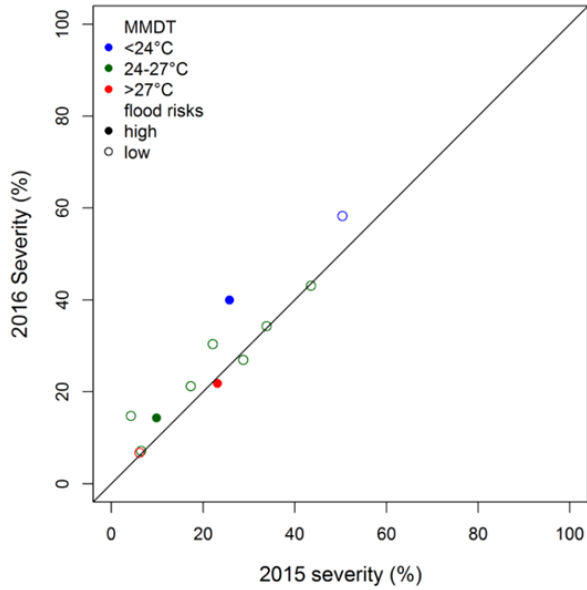


Fig. 3: Evolution of ash dieback severity in 2015-16, summer 2015 MMDT and flood risks, for the 12 sites analyzed. Points above the line had an increase in ash dieback severity between 2015 and 2016.

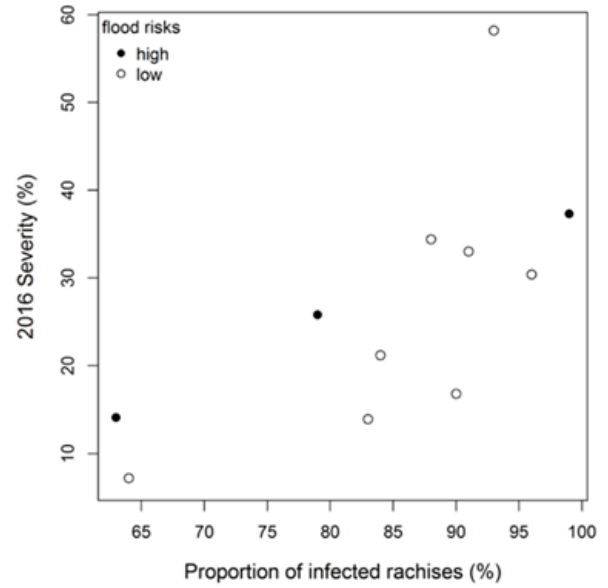


Fig. 4: Ash dieback severity in 2016 against the proportion of infected rachises observed in the litter in 2016 and flood risks.

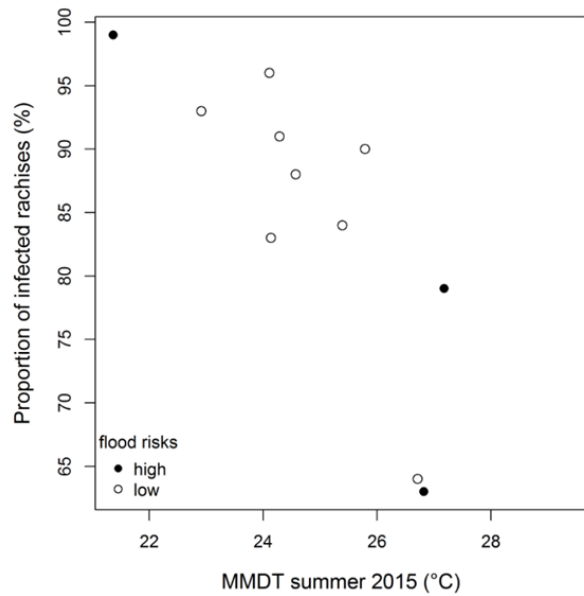


Fig. 5: Proportion of infected rachises in 2016 against summer 2015 MMDT and flood risks

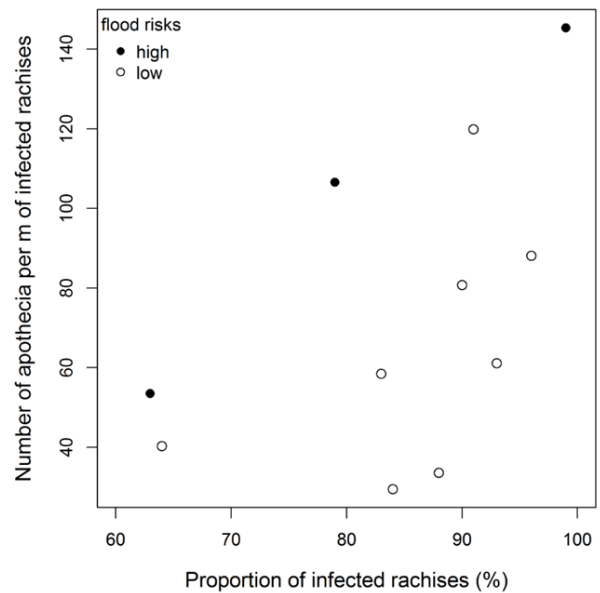


Fig. 6: Number of apothecia observed in 2016 against the proportion of infected rachises observed in the litter in 2016, summer 2015 MMDT and flood risks





Ash dieback severity in 2016 could be positively explained by the ash dieback severity of the previous year ( $p$ -value < 0.01) and negatively by the 2015 MMDT ( $p$ -value < 0.01). The FRI was not significantly related to ash dieback severity ( $p$ -value = 0.14) (Figure 3). Moreover, disease severity in 2016 was positively related to the proportion of infected rachises observed in the litter the same year ( $p$ -value < 0.01) (Figure 4). An indirect effect of the 2015 MMDT on dieback 2016 could be computed by multiplying both direct effects of the 2015 MMDT on the proportion of infected rachises and the effect of the proportion of infected rachises on 2016 dieback. The indirect effect of the 2015 MMDT on 2016 dieback (-2.28) was higher than the direct effect (-0.15). The total effect of the 2015 MMDT on 2016 dieback was the sum of direct and indirect effects (-2.43).

The proportion of infected rachises observed in the litter in 2016 could be negatively related to the 2015 MMDT ( $p$ -value < 0.01). The FRI did not influence the proportion of infected rachises ( $p$ -value = 0.73) (Figure 5). The number of apothecia per m of infected rachises in 2016 could be positively explained by the proportion of infected rachises observed in the litter the same year ( $p$ -value < 0.01) and the FRI ( $p$ -value < 0.01) (Figure 6). The proportion of collar cankers per site could not be explained by the FRI ( $p$ -value 0.99), but was positively related to the number of years with disease presence ( $p$ -value < 0.01).

## Discussion

Structural Equation Modelling with the piecewise package using standardized correlations as path coefficients enabled us to directly interpret the strength of the causal relationship between two variables (Lefcheck, 2016). Overdispersion parameters of beta regression used in SEM were always higher than 1, reflecting excess data variations. The variations could be interpreted either as variability caused by the sampling area, which was large, and by the number of sites studied, which was small, or by non-measured factors such as host density or different levels of susceptibility of trees connected to the two different host species observed.

Our Structural Equation Modelling analysis showed that the severity of dieback induced by *H. fraxineus* was explained by both climate of the previous summer and previous dieback severity. Overall, ash dieback severity remained stable in 2016 compared to 2015, with some trees or sites even showing signs of recovery. This lack of change in tree conditions could be explained by the negative effect of high 2015 summer temperatures on the pathogen. Indeed, several authors have shown that high temperatures strongly affect *Hymenoscyphus fraxineus* survival (Dal Maso and Montecchio, 2014; Hauptman et al., 2013). In 2015, France experienced a scorching summer, and temperatures higher than 35°C that are lethal for *H. fraxineus* were reached at some of the sites during this season. High summer temperatures could affect the pathogen by either limiting foliar infection or by preventing its growth from the rachis to the shoot. Our data support both mechanisms because the 2015 MMDT had a direct action on both the proportion of infected rachises and dieback severity in 2016, and an indirect action on dieback in 2016 via the proportion of infected rachises. Even though we only had indirect measurements of 2015 foliar infection, our data suggest that high summer temperatures had a greater effect on foliar infection than on fungus growth to the shoot. The proportion of infected rachises in the litter is in fact an imperfect indicator of the foliar infections that occurred in the previous summer, for several reasons. First, infected rachises have been shown to remain up to 5 years in the litter (Kirisits, 2015). Moreover, the fungal pseudo-sclerotial plate, which protects infected rachises, could limit rachis degradation with faster degradation of healthy rachises as a consequence. The second possibility is that significant foliar infection occurred during the summer of 2015 but that the fungus grew poorly from the leaves to the shoots, which would have limited tree dieback. This phenomenon may have been enhanced by the very hot 2015 summer, which could explain why at some sites, we observed a very high proportion of infected rachises, while crowns remained relatively healthy. Nevertheless, recovery of the ashes at some of the sites could have been related to the high temperatures experienced during summer 2015. Moreover, summer 2014 was warm enough at some of the sites to negatively affect pathogen development in 2014–15 and the dieback severity in 2015, despite recorded temperatures not reaching 35°C at any of the sites. This could be related to the fact that temperatures experienced by the pathogen in leaves exposed to sun can be above air temperature by up to 20°C (Bernard, 2013; Smith, 1978). As a result, leaf temperature could be a limiting factor for *H. fraxineus* survival, more often than indicated by the meteorological data.

Litter humidity, which we estimated by the flood risk index, influences inoculum production, confirming findings reported in the literature (Gross et al., 2012; Havrdová et al., 2017). With higher inoculum production at high flood risks, the collar of the ash trees should be subject to increased inoculum pressure and more collar infections should be observed after a few years of disease presence at these sites (Enderle et al., 2017; Husson et al., 2012; Marçais et al., 2016). However, the effect of this factor was not significant, perhaps because the flood risk is not a very good proxy, or alternatively, because many sites were only recently infected.

The number of apothecia per length of infected rachis increased with the proportion of infected rachises in the litter. This could be explained by a higher likelihood of the presence of the two different mating types, as conidia are dispersed in the litter via splashing, probably at short distances. Conidia have in fact been shown to act as spermatia (Gross *et al.*, 2012). Gross *et al.* (2012) showed that the different apothecia present on a single rachis generally have different genotypes, suggesting that they result from the arrival of different conidia. *H. fraxineus* fructification could be limited to a greater extent at low pathogen densities (disease front or low host density).

A large unexplained variability was observed for ash dieback severity. This may be linked to the history of invasion in each site that was only indirectly documented using the DSF data. Other large sources of variation were the host density and susceptibility that we did not document. Host density and connectivity with neighboring host stands maybe a strong factor affecting disease severity (Havrdová *et al.*, 2017; Skovsgaard *et al.*, 2017). We did not measure it for lack of time. However, just 2 sites were pure ash stands, and these 2 were of limited surface; most stands were edges with only part of the population being ash and thus presumably at low host density. Also, in the studied area *F. angustifolia* is mixed with *F. excelsior* and the 2 species coexisted in most our sites. Although the 2 species have been described as susceptible (Drenkhan and Hanso, 2010; Kirisits *et al.*, 2010), it has been shown that the level of susceptibility was variable between and within ash species. (Diminić *et al.* 2017, Drenkhan *et al.* 2015, Hauptman *et al.* 2016).

In this study, the negative impact of high summer temperatures on *H. fraxineus* was confirmed in the field. Climate change has usually been studied only for its adverse effects on plant diseases. Here, we described an example where its effect may be favorable, by limiting the impact of a devastating invasive disease. This outcome has already been predicted for several invasive species such as fish and plants, but also tree pathogens. For example, the brook trout (*Salvelinus fontinalis*) population decreased with temperatures increases, as a result of brown trout (*Salmo trutta*) being a superior competitor at warm temperatures (Rahel and Olden, 2008). *Phytophthora ×alni* is susceptible to high summer temperatures which leads to alder recovery during hot summers (Aguayo *et al.*, 2014). Engler *et al.* (2009) and Hellmann *et al.* (2008) showed that climate change could induce dispersal limitations, affect invasive species spread across a landscape, and therefore affect the distribution of existing invasive species. Our results suggest that the severity of ash dieback may be mitigated in the south of France by high summer temperatures. In agreement with this, the disease spread only marginally in the area in 2016–17 according to data from the DSF, while elsewhere it has spread at a relatively regular speed of about 50 km per year until now, including in the west of France in 2016–17. The disease should already have been observed throughout our sampling area, but no symptoms or infected rachises were found in the two most southern sites. It is unclear whether the fungus will develop in this southern part of France in the future. Goberville *et al.* (2016) predict that with expected climate change, *H. fraxineus* could disappear from France as unsuitable warm conditions will restrict its ability to cause disease.

## Acknowledgements

We greatly appreciate the valuable assistance provided by A. Gilet and O. Cael. This work was supported by grants from the French Forest Health Department, the French Ministry of Agriculture and Forestry, the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), and the BiodivERsA EU project RESIPATH. The UMR1136 research unit is supported by a grant managed by the French National Research Agency (ANR) as part of the “Investissements d’Avenir” program (ANR-11-LABX-0002-01, Laboratory of Excellence ARBRE).

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