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Temporal evolution of collar lesions associated with ash dieback and the occurrence of *Armillaria* in Belgian forests

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Summary

Ash dieback, caused by the fungus *Hymenoscyphus fraxineus,* has been observed in Europe for several years. In Belgium, the disease was first reported in 2010. Besides crown defoliation and dieback, collar lesions have sometimes been reported. To evaluate the prevalence and the progression of collar lesions and crown defoliation in ash dieback-affected stands of various ages, a survey was conducted in 2013 and 2014 on 268 ash trees (*Fraxinus excelsior*) originating from 17 Walloon forest stands. The results showed that the proportion of trees with collar lesions greatly increased between June 2013 and September 2014 and that there appeared to be no significant link between a tree's diameter-at-breast height (DBH) and collar lesion occurrence. The mean percentage of defoliation increased in each forest stand across time, with observations conducted in September 2013 and 2014 showing a positive correlation with the mean percentage of trees with collar lesions. Molecular tests were carried out on 103 additional trees originating from 12 of the 17 stands to evaluate the occurrence of *H. fraxineus* and *Armillaria* spp. at the collar level. Most of the trees (98%) were infected by *H. fraxineus*. In contrast, only 41% of the samples were infected with *Armillaria* spp., most commonly *A. gallica* and *A. cepistipes*. This study discusses the role of *Armillaria* spp. and the rapid increase in the number of trees with collar lesions within the context of the evolution of ash dieback in Europe.

1 Introduction

In the early 1990s, severe dieback in common ash (*Fraxinus excelsior*) was reported in Poland and Lithuania. In 2006, a fungus, *Chalara fraxinea*, was identified as the primary causal agent (Kowalski 2006). Its sexual stage was described in 2009 and initially assigned to *Hymenoscyphus albidus* (Kowalski and Holdenrieder 2009), a long-known fungus non-pathogenic to *Fraxinus excelsior* in Europe (Husson et al. 2011; Gross and Sieber 2015). In 2011, molecular studies revealed that two cryptic species were present on blackened parts of ash leaves. One was *H. albidus* and the other was an aggressive pathogen described as a new species, *Hymenoscyphus pseudoalbidus*, (Queloz et al. 2011), but recently renamed *Hymenoscyphus fraxineus* (Baral et al. 2014).

The pathogen has been reported in Korea (Han et al. 2014), Japan (Zhao et al. 2012) and China (Zheng and Zhuang 2014) where it occurs as a harmless saprotroph on native ash trees, suggesting that it has been introduced to Europe from Asia (Baral and Bemmann 2014).

Since its first identification, *H. fraxineus* has been reported throughout Europe (Timmermann et al. 2011; Gross et al. 2014), causing a massive decline in the number of ash trees (Pautasso et al. 2013; McKinney et al. 2014), and a decrease of ash regeneration in ash stands with long disease history (Lygis et al. 2014). In Belgium, the pathogen was first detected in 2010 (Chandelier et al. 2011) and the disease is present throughout the country, notably in Wallonia where ash trees are common in the forests (Alderweireld et al. 2015).

The apothecia are produced in the leaf litter on ash petioles that have been infected in previous years (Kirisits and Cech 2009; Gross et al. 2012). Ascospores are wind-dispersed in summer, generally from June to early September (Timmermann et al. 2011; Hietala et al. 2013; Chandelier et al. 2014). Occasionally, ascomata develop on dead shoots (Kowalski 2006). Infected trees exhibit a wide range of symptoms, including brown spots on the leaves and leaf stalks, wilting and premature leaf shedding, brown-to-orange bark necrosis on shoots, dead shoots and branches, a proliferation of epicormic shoots, bark cankers and wood discoloration (Cech 2006; Bakys et al. 2009). Collar lesions have also been reported (Skovsgaard et al. 2010; Husson et al. 2012; Enderle et al. 2013), but in many field surveys of ash dieback in Europe, this symptom has rarely been described, although Bakys et al. (2011) showed a correlation between collar rot and ash dieback intensity. *Armillaria* spp. have been detected on most of the necrotic tissues (Bakys et al. 2011; Husson et al. 2012; Enderle et al. 2013), and their role as secondary pathogens in the decline of ash trees has been shown (Bakys et al. 2011; Husson et al. 2012).

The aims of this study were to evaluate the prevalence and progression of collar lesions and crown defoliation in ash dieback-affected stands of various ages and the association of *Armillaria* spp. and *H. fraxineus* with collar lesions. The study was carried out in the Walloon region of Belgium.

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2 Material and methods

2.1 Study sites and field observations

In June 2013, 268 ash trees in 17 forest stands were selected in Wallonia. *Hymenoscyphus fraxineus* was isolated from ash regeneration in these forest sites in 2010 (stands 1, 2 and 3), in 2011 (stands 4–13) or in 2012 (stands 14–17). The forest stands were chosen randomly in the area most suited to ash production (Anonymous, 1991) and with the highest density of ash trees (Fig. 1). They represented different types of forest (plantation or natural regeneration, pure or mixed forest). They also differed in terms of soil moisture characteristics based on a 9-level scale (from 1 = high soil drainage to 9 = poor soil drainage) established for soils in Wallonia (Anonymous, 2007) (Table 1). In each stand, *Fraxinus excelsior* trees with a minimum diameter-at-breast height (DBH) of 10 cm were randomly selected among dominant or co-dominant trees to facilitate the visual observations of symptoms in the crown. The selected trees were placed in five DBH categories (1 = 10–15 cm; 2 = 15–25 cm; 3 = 25–35 cm; 4 = 35–45 cm; and 5 = >45 cm). The number of trees in each stand varied from 10 to 20 (Table 1). Most of the selected trees were young or immature (only six had a DBH larger than 55 cm). Observations were conducted over two periods (June and September) in each year of the survey (2013 and 2014). Four parameters were measured: DBH; level of crown defoliation in 5% steps, in line with United Nations Economic Commission for Europe protocol (UNECE 2006); occurrence of collar lesions (presence/absence); and lesion size on the collar (percentage of affected collar circumference of each tree). This last parameter was measured only in June.

2.2 Statistical analyses

To test whether the occurrence of collar lesions varied according to DBH category (Test 1) or type of forest (pure *vs.* mixed forest) (Test 2), we used mixed-effects logistic regression, with the presence/absence of collar lesion as the response variable, the DBH category or the type of forest as the fixed explanatory variables and the forest site as the random effect. Data from the four survey periods were analysed separately. The significance of the DBH category or forest type effect was tested using a likelihood ratio test (LRT). For the first survey period (June 2013), the data corresponding to the last DBH category were removed prior to Test 1 because the absence of trees with collar lesions in this category caused model instability. The mean defoliation level during the four survey periods was compared using a linear mixed-effect model, with the site and the trees within that site considered as random effects. An LRT was carried out to determine the survey period fixed effect. The mean percentages of affected collar circumference in the five DBH categories were compared using a linear mixed-effect model after arcsine transformation of the variable, with the forest site as the random effect. An LRT was carried out to determine the DBH category fixed effect. All pairwise post hoc multiple comparisons were performed using the R multcomp package default method (Hothorn et al. 2008; Bretz et al. 2010).

Separately for each period, we calculated the Pearson correlation coefficient between the percentage of trees with collar lesions per site and the mean defoliation level per site. To test the significance of these correlations, permutation tests were carried out with 1000 permutations and two-tailed tests (significance level set at $p = 0.05$ in all cases).

All the statistical analyses were performed using R software (version 3.2.1, 2015) and the lme4 package for the mixed models (Bates et al. 2013).

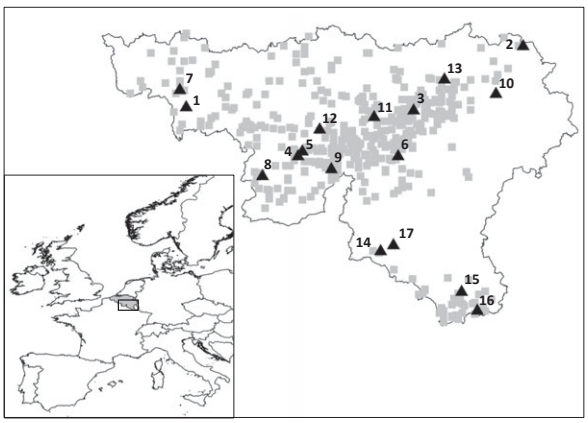

Fig. 1. Density of *Fraxinus excelsior* (grey squares) and location of the 17 forest stands selected for the survey of collar lesions in ash trees (black triangles), in the Walloon region (southern Belgium).

Table 1. Evolution of the number of ash trees with collar lesions over two years (2013–2014) in 17 ash dieback-affected forest stands in Wallonia (N = number of trees examined per stand).

No.	Location	Region	Stand origin	Forest type	Soil moisture[1]	Mean DBH ± SD[2]	N	2013		2014	
								June	Sept	June	Sept
1	Hainin	Hesbaye	Plantation	Pure	7	17.2 ± 3.6	18	16	16	16 (3)[3]	16 (3)[3]
2	La Calamine	Hesbaye	Plantation	Pure	8	20.2 ± 2.8	20	17	18	20	20
3	Modave	Condroz	Natural regeneration	Pure	2	29.4 ± 6.1	20	17	18	18	18
4	St Lambert	Fagne	Plantation	Pure	5	19.2 ± 2.5	20	7	9	12	12
5	Minières	Fagne	Natural regeneration	Pure	5	45.6 ± 9.2	10	0	2	9	9
6	Les Halleux	Famenne	Plantation	Pure	2	15.7 ± 2.1	15	0	0	0	0
7	Beloeil	Hesbaye	Natural regeneration	Mixed	8	39.8 ± 8.0	15	3	3	4	5
8	Rance	Fagne	Plantation	Pure	5	18.7 ± 3.2	15	2	2	2	2
9	Gochenée	Fagne	Natural regeneration	Pure	2	34.0 ± 5.9	15	5	8	10	11
10	Spa	Condroz	Natural regeneration	Pure	2	29.7 ± 3.0	15	3	8	13	14
11	Gesves	Condroz	Natural regeneration	Mixed	4	33.7 ± 7.3	15	1	2	6	6
12	Devant-Les-Bois	Condroz	Natural regeneration	Mixed	5	38.7 ± 14.6	15	1	1	3	4
13	Seraing	Condroz	Plantation	Pure	4	23.9 ± 2.4	15	0	0	0	0
14	Senseruth	Ardennes	Plantation	Mixed	2	22.9 ± 2.2	15	8	10	12	12
15	Buzenole	Ardennes	Natural regeneration	Pure	2	38.6 ± 7.8	15	0	0	3	3
16	Musson	Gaume	Natural regeneration	Mixed	2	41.5 ± 10.8	15	1	1	7	8
17	Bertrix	Gaume	Plantation	Pure	2	15.8 ± 2.1	15	3	4	9	10
Total							268	84	102	144	150
(%)								31.3	38.1	53.7	56

[1]Soil moisture based on a 9-level scale (1 = high drainage; 9 = very low drainage).
[2]DBH expressed in cm (mean ± SD) and measured in June 2013.
[3]In brackets = number of dead trees among the trees with collar lesions.

2.3 DNA-based detection of *H. fraxineus* and *Armillaria* spp

2.3.1 Detection of H. fraxineus and Armillaria *spp. in the collar lesions*

In order to prevent any artificial infection from experimental wounding of the trees surveyed for collar lesion development, the wood and bark samples to be used for DNA-based diagnostics were collected from the collar lesions of additional trees (N = 103). These trees were selected in February 2014 in 12 of the 17 forest stands, most of them being stands with the greatest occurrence of collar lesion in September 2013.

The trees were chosen to represent four DBH categories (Table 2) and did not show any epicormic shoots, wounds or external signs of *Armillaria* (no raised bark with a visible mycelial fan on the inner bark) at their base. In order to limit the wound induced in the sampled trees and to target the necrotic area, a drill was inserted in the centre of the lesion (0.5 cm drill diameter; 4 cm drilling depth; ~10 cm above the ground) and the sawdust (outer bark, inner bark and sapwood, ~200 mg plant material) was collected in a 1.5-ml tube. The drill was carefully disinfected with alcohol at 70° between each sampling. DNA was extracted from the sawdust using a commercial kit (High Pure PCR Template Preparation Kit, Roche Diagnostic, Germany) after incubating the plant material for 1 h 30 min at 55°C in 250 µl lysis buffer (Tris-HCl pH8.0 100 mM; EDTA 20 mM; NaCl 1.4 M; CTAB 2%; PVP K-30 2%) and 40 µl proteinase K (1 mg/ml). Two detection tests were conducted on the DNA extracts. The detection of *H. fraxineus* was performed using real-time polymerase chain reaction (PCR) (Chandelier et al. 2010) with a StepOnePlus thermocycler (Life Technologies), an annealing temperature of 60°C, a normalized fluorescence threshold of 0.2 and a qPCR Core kit (Eurogentec, Liège, Belgium). The nested PCR-restriction fragment length polymorphism (RFLP) method developed by Lochman et al. (2004) using Platinum Taq DNA polymerase (ThermoFisher Scientific, Erembodegem, Belgium) was used to identify species of *Armillaria*. PCR products digested with the restriction enzyme *HinfI* (Invitrogen™; Merelbeke, Belgium) were separated by electrophoresis in 2% agarose gels stained with GelRed and compared with the restriction profile of reference *Armillaria* strains provided by Dr Marçais [Institut national de la recherche agronomique (INRA), Nancy, France].

2.3.2 Detection of Armillaria *spp. in forest soil*

An auger was used to collect soil samples (with roots and eventually rhizomorphs) from the area around 74 of the 103 additional trees selected in each of the 12 stands. The objective was to determine the population of *Armillaria* in the soil

Table 2. Number of trees (sawdust collected in the lesion at the base of the trunk) infected by *Hymenoscyphus fraxineus* alone (*H. frax.* alone), by *Armillaria* spp. alone (Armil. alone) or by both pathogens (H. frax. + Armil.) of 103 trees categorized according to DBH (N = number of trees selected in each DBH category) in 2014. The *Armillaria* spp. identified are indicated (Ag = *A. gallica*; Ac = *A. cepistipes*, Am = *A. mellea*; Ab = *A. borealis).*

DBH categories	N	Number of stands	Mean DBH ± SD	*H. frax.* alone	*H. frax* + Armil.	Armil. alone	Ag	Ac	Am	Ab
8–10 cm	18	8	8.0 ± 1.3	15	3	0	3	0	0	0
10–15 cm	28	10	12.7 ± 1.5	17	11	0	7	3	1	0
15–25 cm	37	12	20.6 ± 2.5	22	14	1	9	6	0	2[1]
>25 cm	20	9	30.5 ± 5.8	7	12	1	8	5	0	1[2]
Total (%)	103			61 (59%)	40 (39%)	2 (2%)	27 (60%)	14 (31%)	1 (2%)	3 (7%)

[1]In association with *A. cepistipes*.
[2]In association with *A. gallica*.

of each stand (not specifically in the roots of the sampled trees). The soil samples were collected ~1 m from the tree trunk. The auger was disinfected with alcohol at 70°C between each sampling. DNA was extracted as for the sawdust, after grinding the root tissues/rhizomorphs with liquid nitrogen using a pestle and mortar. The presence of *Armillaria* spp. was determined as described earlier.

2.3.3 Quantification of H. fraxineus *DNA in the wood*

To prevent the wood and pith samples being compromised by DNA from conidia residing on bark surface, *H. fraxineus* DNA levels at the collar were determined in four trees felled at two locations near the stands under study (Hainin and Modave, Table 1) in February 2014: here, the sawdust samples were collected from the discoloured outer sapwood close to the bark necrosis and from the pith using a drill that was disinfected between each subsampling. DNA extraction and real-time PCR were carried out as mentioned earlier. The cycle threshold (C_t) values were converted into log-transformed DNA concentrations (expressed in pg per PCR) using a standard curve established from four serial dilutions of DNA (20 000, 2000, 200 and 20 pg) prepared in duplicate and extracted from the mycelium of the pathogen (isolate 4341 originating from Belgium, characteristics of the standard curve: slope = −3.817, constant = 34.697; R^2 = 0.9998). The DNA concentration in each DNA extract was measured with a spectrophotometer (Eppendorf BioSpectrometer, Rotselaar, Belgium) and normalized at the same concentration (1 ng/μl) prior to the PCR.

3 Results

3.1 Prevalence and temporal evolution of collar lesions

In June 2013, 84 trees (31.3% of the sampled trees) showed collar lesions. In September 2014, 150 trees (56%) showed collar lesions among which three were dead trees (Table 1). The five most affected stands in September 2014 (stands 1, 2, 3, 5 and 10 where at least 90% of the trees had collar lesions or were dead) were in three regions of the country (Fig. 1, Table 1) with contrasting levels of soil moisture (Table 1). The disease was first reported in 2010 in three of these highly infected stands. The three dead trees identified at the end of the survey had grown in stand 1, where trees with collar lesions had first been detected in 2012. There were two forest sites (stands 6 and 13) where the selected trees did not develop any collar lesions, although the pathogen had been detected at these sites in 2011 on ash regeneration.

As shown in Fig. 2a, trees with collar lesions were observed in all DBH categories in June 2014, but no tree in the last DBH category (>45 cm) had this symptom in June 2013. The proportion of trees with collar lesions increased in all DBH categories between the 2 years. The analysis of the mean percentage of affected collar circumference confirmed the rapid progression of basal lesions in all the DBH categories between 2013 and 2014 (Fig. 2b). On average, about 16% of the collar circumference was affected in June 2014. There was no significant link between DBH or forest type and collar lesions occurrence in the four survey periods (Table 3). In contrast, the proportion of the circumference with collar lesions differed significantly among the DBH classes (LR = 13.96, df = 4, p = 0.007). However, none of the post hoc pairwise comparison tests was significant at the 0.05 alpha level.

3.2 Seasonal evolution of crown defoliation and relationship with collar lesion

Defoliation intensity differed significantly in the four survey periods (LR = 337.25; df = 3; $p < 0.001$), with the defoliation measured in September being consistently higher than that measured in June of the same year. In 2014, there were higher levels of mean defoliation in both periods (June and September) than in the corresponding periods in 2013 (Fig. 3a). There was a positive correlation between the percentage of trees with collar lesions and the mean level of defoliation in each of the 17 forest stands in September 2014 (R = 0.53, p = 0.03) (Fig. 3b) and, to a lesser extent, in September 2013 (R = 0.49, p = 0.04). Such correlations were not significant in June (R = 0.27, p = 0.33 in 2013 and R = 0.43, p = 0.09 in 2014). There were only three trees without collar lesions and with <5% crown defoliation in September 2014; one was at a site that

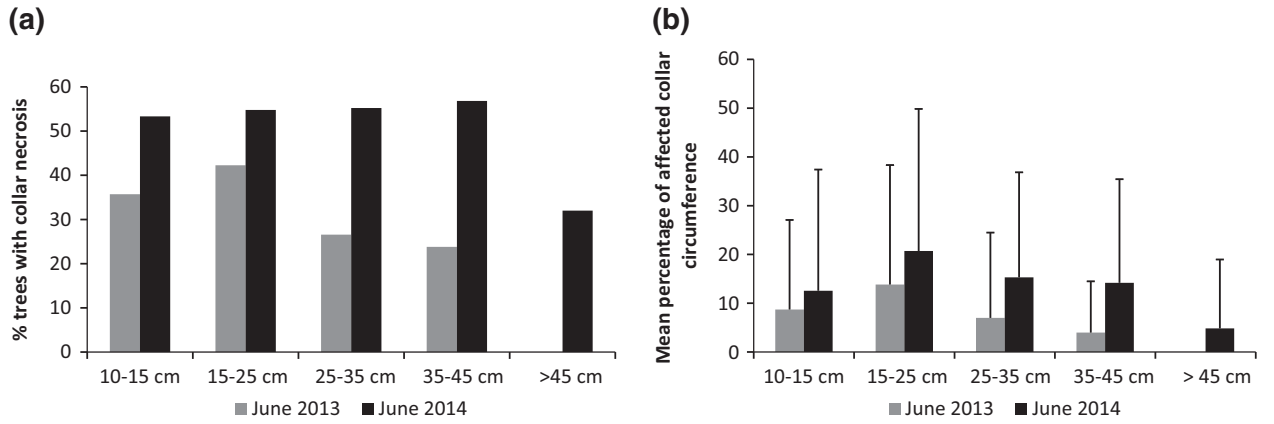

Fig. 2. Proportion of ash trees with collar lesions (a) and mean percentage of affected collar circumference (b) according to the year the survey was conducted and the DBH of the trees.

Table 3. Probabilities (p) associated with the likelihood ratio test (LR) carried out to evaluate the link between collar lesion occurrence and DBH category (Test 1) or forest type (pure or mixed forest) (Test 2) for the four survey periods.

Period	Variable	LR	df	p
June 2013[1]	Forest type	0.27	1	0.60
	DBH	2.63	3	0.45
September 2013	Forest type	0.62	1	0.43
	DBH	2.57	4	0.63
June 2014	Forest type	0.32	1	0.57
	DBH	5.55	4	0.23
September 2014	Forest type	0.23	1	0.63
	DBH	6.50	4	0.16

[1]Data corresponding to the last DBH category removed prior to Test 1 because the absence of trees in this category caused model instability.

had been infected in 2011 (stand 6, DBH in September 2014 = 18.8 cm) and the other two were in stands infected in 2012 (stands 12 and 13, DBH in September 2014 = 24.2 and 26.7 cm, respectively).

3.3 Occurrence of *H. fraxineus* and *Armillaria* spp. in collar lesions

As shown in Table 2, H. *fraxineus* was detected in all but two samples of sawdust collected within the necrotic lesions at the base of the trunk (98% of samples), whereas *Armillaria* spp. were detected in 42 samples (41%). Among the trees infected with *Armillaria* spp., 40 (95%) were also infected with *H. fraxineus*. The proportion of trees infected with *H. fraxineus* alone ranged from 83% (<10 cm DBH) to 35% (>25 cm DBH) (Table 2).

Armillaria gallica and *A. cepistipes* were the most frequent *Armillaria* species identified (60% and 31% of the total number of samples infected with *Armillaria* spp., respectively) (Table 2). *Armillaria gallica* was the species identified in the two trees infected only with *Armillaria* spp. One tree was infected with *A. mellea,* and three were infected with *A. borealis* in combination with *A. cepistipes* (two trees) or *A. gallica* (one tree). The three trees where *A. borealis* was detected came from the same stand (stand 10, Fig. 1), located in the coldest region of the country.

No *Armillaria* spp. were detected in stand 1 at the collar level of the 12 sampled trees, but *A. gallica* and *A. cepistipes* were detected in the soil of this stand (Table 4). *Armillaria gallica* was detected in the soil from 11 of the 12 stands, and *A. cepistipes* was detected in seven of the stands. The species *A. borealis*, identified at the collar base of trees from stand 10, was also detected in the soil from this stand and from stand 6. The proportions of *A. gallica* and *A. cepistipes* in the soil were similar to those found in the necroses at the collar base (Table 4).

3.4 Presence of *H. fraxineus* in the wood at the collar base

The presence of *H. fraxineus* was detected at the collar base in the discoloured wood of the four trees analysed, as well as in the pith when it was in contact with the discoloured wood. The DNA concentration in the non-discoloured wood or in the pith when not in contact with the discoloured wood was nil or very low (Table 5).

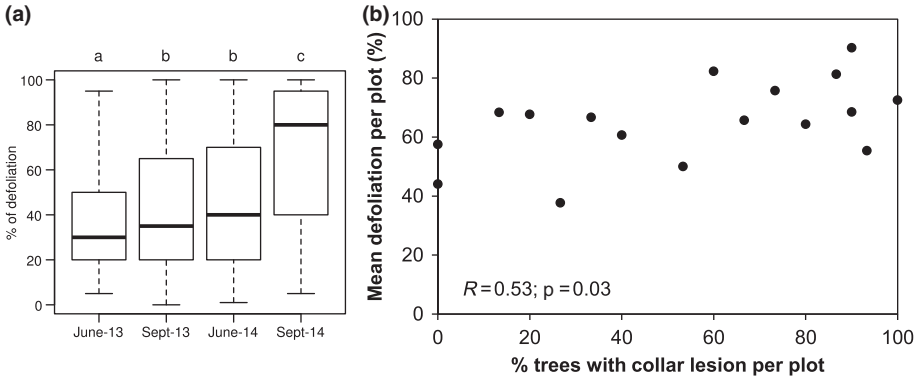

Fig. 3. (a) Percentage of tree defoliation during the four survey periods (from June 2013 to September 2014). Different letters represent periods with a significantly different mean percentage of defoliation (alpha = 0.05). (b) Correlation between mean defoliation and number of trees with collar lesions per plot in September 2014.

Table 4. Comparison between the *Armillaria* spp. identified at the collar base and in the soil around a subsample of the selected trees from 12 ash stands (Ag = *A. gallica*; Ac = *A. cepistipes*, Am = *A. mellea*; Ab = *A. borealis*).

		Collar base						Soil/Root					
Stand no.	Location	N	Trees with *Armillaria*	Ag	Ac	Am	Ab	N	Soil with *Armillaria*	Ag	Ac	Am	Ab
1	Hainin	12	0	0	0	0	0	9	4	3	1	0	0
2	La Calamine	14	5	0	5	0	0	9	1	0	1	0	0
3	Modave	12	3	2	1	0	0	9	3	2	1	0	0
4	St Lambert	10	4	1	3	0	0	7	5	4	1	0	0
6	Les Halleux	5	4	4	0	0	0	4	4	3	0	0	1
7	Beloeil	8	4	4	0	0	0	5	3	3	0	0	0
9	Gochenée	4	3	2	1	0	0	4	3	1	3	0	0
10	Spa	9	5	2	3	0	3	7	6	1	2	0	5
14	Senseruth	12	6	5	0	1	0	7	5	5	0	0	0
15	Buzenole	4	2	2	0	0	0	4	1	1	0	0	0
16	Musson	6	5	5	0	0	0	4	4	4	0	0	0
17	Bertrix	7	1	0	1	0	0	5	5	2	3	0	0
Total		103	42	27	14	1	3	74	44	29	12	0	6

4 Discussion

4.1 Cause of collar lesions in ash

By carefully selecting trees without epicormic shoots at the stem base, which are a typical entry point for *H. fraxineus* in trees (Kowalski 2006; Gross et al. 2014), and using a sampling procedure that specifically targeted the necrotic area at the base of the trunk, we demonstrated that, in forest stands where *H. fraxineus* had been detected a few years earlier, this pathogen could infect ash trees of various ages via the bark, even trees with a DBH higher than 25 cm. The detection of *H. fraxineus* from the bark to the pith in the affected collar lesions also provided evidence of the pathogen's entry through the bark at ground level. These results confirm those of Husson et al. (2012) who concluded that *H. fraxineus* was involved in the collar lesion induction. *Armillaria* was associated with *H. fraxineus* at the collar base in 39% of the cases, indicating its major role in ash dieback, as reported in other studies (for a review, Gross et al. 2014). None of the sampled trees displayed external symptoms of *Armillaria* infection (raised bark at the collar base or fruiting bodies), suggesting that the occurrence of *Armillaria* can be underestimated in surveys based on visual observation. In only 2% of the selected trees did *Armillaria* spp. occur without *H. fraxineus*. The main *Armillaria* species identified, *A. gallica* and *A. cepistipes*, had been identified in other studies (Bakys et al. 2011; Husson et al. 2012; Enderle et al. 2013). These species are common in Europe (Guillaumin et al. 1993; Termoshuizen and Arnolds 1994) and are adapted to rapid soil colonization due to their rhizomorph growth habit (Morrison 2004). They are therefore considered as secondary pathogens infecting weakened trees, which is consistent with their secondary role in the ash dieback infection process. The analysis of the population of *Armillaria* species found in the soil in each of the 12 forest stands in this study confirmed the results obtained from samples collected at the stem base, with a higher proportion of *A. gallica*, followed by *A. cepistipes*. All the sampled stands were infected with *A. gallica* and/or *A. cepistipes*.

Table 5. DNA concentration of *H. fraxineus* measured in the wood and pith at the collar base of four infected trees, using real-time PCR.

Location	Tree (age)	Material	DNA[1]
Hainin	H1 (14)	Discoloured wood	91
		Pith (contact with discoloured wood)	310
	H2 (14)	Discoloured wood	2919
		Pith (contact with discoloured wood)	283
		Non-discoloured wood	0
Modave	M1 (7)	Discoloured wood	135
		Pith (no contact with discoloured wood)	0
	M2 (7)	Discoloured wood	1112
		Pith (contact with discoloured wood)	1450
		Non-discoloured wood	5

[1]Expressed in pg/PCR and based on a standard curve established from serial dilutions of DNA extracted from mycelium of isolate 4341 of *H. fraxineus* (CRA-W collection).

Only three of the 268 trees surveyed (1.1%) did not display any symptoms of ash dieback. This result confirms the finding reported by other studies that the percentage of ash trees potentially resistant to the disease is < 5% (Husson et al. 2012; McKinney et al. 2014).

4.2 Influence of inoculum pressure and DBH of the tree on the occurrence of collar lesions

Our study highlighted varying proportions of ash trees with collar lesions both among and within stands. There were two stands where none of the sampled trees developed collar lesions at least 3 years after the pathogen had first been detected in these stands. Given that the infection occurs via the bark at the collar base (this study; Husson et al. 2012) and that there is a huge concentration of ascospores of the pathogen near the ground (Chandelier et al. 2014), this variation in the response of trees depending on the stand is consistent with studies that have shown that inoculum pressure at ground level can differ greatly, depending on the stand (Kowalski et al. 2013) or on different areas within the stand (Chandelier et al. 2014). This variation in inoculum pressure at ground level could result from ash density within the stand, which influences the amount of infected rachis in the litter. However, in our study, the proportion of trees with collar lesion was not significantly different in mixed or pure stands. It could also be linked to stand characteristics that limit the production of ascospores at ground level. Apart from humidity during the infection process, as pointed out by Keßler et al. (2012) and Kowalski et al. (2013), the presence of plants forming a dense cover at ground level could affect the intensity of the disease by modifying the amount and quality of light reaching the ground and interfering with ascospore maturation or discharge. The accumulation of infected rachis over several years is also worth considering. Gross and Holdenrieder (2013) and Kirisits (2015) have shown that the apothecia could be produced over several years (at least 5 years in the latter study) on infected rachis covered by pseudosclerotia in the litter. This hypothesis is plausible as most of the stands in our study that were more severely affected by infection at the collar base were also those where the pathogen had first been reported in 2010.

Statistical analyses did not reveal any significant effect of the tree DBH on the occurrence of collar lesion (disease incidence). However, from 2013 to 2014, we observed a rapid increase in the proportion of trees with collar lesions in the five DBH categories. Our data also showed a delay in the occurrence of collar lesions in the highest DBH category (DBH > 45 cm) between June 2013 and June 2014. This suggests that a variable critical concentration of ascospores is needed to cause collar rot, depending on the age of the tree. Young trees are characterized by thin bark and active growth, resulting in bark cracks, and they also have a high density of active lenticels. These natural entry points (bark cracks and lenticels) probably facilitate the penetration of *H. fraxineus* at the collar base via the ascospores. As the trees develop, the number of active lenticels decreases, reducing the probability of infection.

4.3 Temporal gap between crown defoliation and collar lesion

Our results showed that the most pronounced increment in crown defoliation (June 2014 – September 2014, Fig. 1) was preceded by the most pronounced increment in collar lesion frequency (September 2013 – June 2014, Table 1), suggesting that collar lesions accelerate the degradation of the health of ash trees. This could lead to the hypothesis that in the first year of infection, defoliation is caused by *H. fraxineus* ascospores originating from other infected stands. After 2 or 3 years, however, defoliation results from new infections in the crown originating from infected sites nearby and from within the stand, as well as from reduced water and nutrient supplies caused by collar lesions affecting the inner bark, cambium and sapwood at the trunk base, a situation that is aggravated by the activity of *Armillaria* species degrading the root system. A positive correlation between mean defoliation level and number of trees with collar lesions per site in September 2013 and 2014 provides evidence of a relationship between crown defoliation and collar lesions. A strong association between crown dieback and collar lesion in ash trees affected by *H. fraxineus* has already been noted by Skovsgaard et al. (2010) or Husson et al. (2012) and is confirmed in this study.

4.3 Guidelines for phytosanitary survey

In both study years, the severity of the disease as indicated by defoliation level increased from June to September. An increase in crown dieback from the beginning to the end of the growing season has already been reported (Bakys et al. 2013). This could result from the production of epicormic shoots in June masking the true defoliation level, these young shoots being rapidly infected during the season and less visible in September. Other factors, particularly meteorological conditions and site characteristics, could also influence defoliation level. Nevertheless, this seasonal variation needs to be taken into account in the identification of ash trees able to resist or tolerate the disease and in the detection of the disease in new areas, because it shows the need to conduct monitoring at the most appropriate period in order to avoid underestimating defoliation due to the production of epicormic shoots or complete defoliation masking crown dieback symptoms.

The high frequency of trees with collar lesions in forest stands is a critical parameter to take into account in assessing trees that are potentially resistant to the disease, especially when grown from scions, because under such conditions defoliation results not only from clone susceptibility, but also from the ability of the pathogen to enter the rootstock.

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