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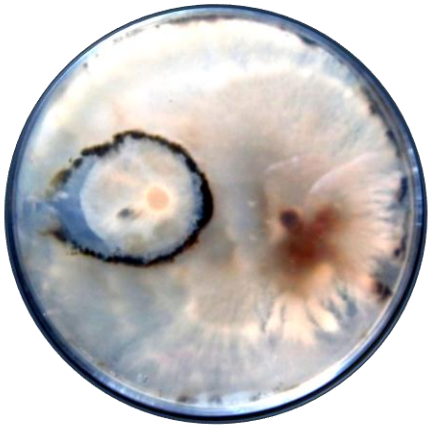
Licence Biologie des Organismes

In vitro interactions between *Hymenoscyphus* species and endophytic fungi from hardy Oleaceae



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Work placement from 10th March to 6th June 2014

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"Le présent rapport constitue un exercice pédagogique qui ne peut en aucun cas engager la responsabilité de l'entreprise ou du laboratoire d'accueil".

Abstract

Hymenoscyphus pseudoalbidus is an invasive alien pathogenic fungus and is responsible for ash dieback in Europe. In contrast, *H. albidus* is a non-pathogenic indigenous, endophytic fungus. In a previous study 34 endophytic fungi were isolated from four hardy woody Oleaceae. In this work, in vitro interactions between *H. pseudoalbidus* and *H. albidus* and these endophytic fungi were determined. Dual cultures were prepared in malt extract agar between the 34 endophytes and 3 isolates of *H. pseudoalbidus* and 2 isolates of *H. albidus*. Four types of interaction were observed: inhibition of the endophyte by *Hymenoscyphus*, inhibition of *Hymenoscyphus* by the endophyte, barrage formation or over-growth. Interactions changed with time. The most common initial interaction was inhibition of the endophyte by *Hymenoscyphus*; followed by an increase in barrage formation and over-growth. *H. albidus* interactions were dominated by over-growth, whereas *H. pseudoalbidus* generally formed barrages, followed by over-growth. Interactions between *H. albidus* and *H. pseudoalbidus* did not show the exclusion of one species over the other but a general growth of both fungi in the presence of the other. Four endophytic fungi (*Plenodomus influorescens*, *Glomerella cingulate*, *Fusarium lateritium*, Sordariomycetes sp) inhibited the growth of all five *Hymenoscyphus* sp. isolates.

Key words: Fungus. Interaction. *Hymenoscyphus pseudoalbidus. Hymenoscyphus albidus* .Endophyte.

Acknowledgements

I would like to thank all the people from University of Aberdeen and especially the Department of Plant and Soil Science, from the Administration office to all the Researchers and the PhD students, for their general friendly welcome and all the time they gave me.

More specifically, I would like to thank my supervisor Professor Steve Woodward of the Department of Plant and Soil Science, University of Aberdeen who gave me the opportunity to work on this project and who provided me guidance during my internship work.

It is impossible for me not to acknowledge Dr.Hedda Weitz for her gentleness and the precious time she gave to help me in my project. Her massive work in the laboratory deserves plenty acknowledge and recognition.

I wish to acknowledge all my family supporting in all my projects. Anywhere I am I know I have them supporting me and for that I am very thankful. My Scottish family is also very important for this project so I acknowledge all my friends in this wonderful country for their support and help during this placement.

Finally, I would like to acknowledge the AQUIMOB plan for the provision of funding for this project, through the office for international relations in Pau.

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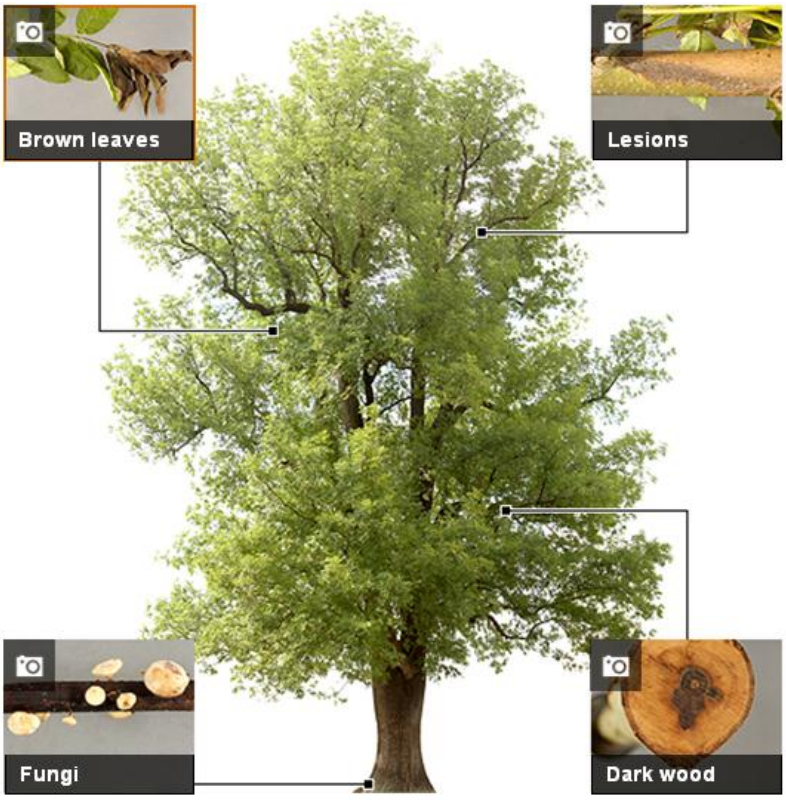


Figure 1: Symptoms of ash dieback (BBC

I] Introduction

Hymenoscyphus pseudoalbidus (anamorph *Chalara fraxinea*) is the causal agent of ash dieback, a disease that has rapidly spread throughout most of the Europe (Junkker *et al*. 2013). This fungus is mainly responsible for population decline of the Oleaceae F*raxinus excelsior* and *Fraxinus angustifolia* but can also infect other ash species such as *F. nigra* (black ash), *F. pennsylvanica* (green ash) and *F. americana* (white ash) *F.mandschurica* (Manchurian ash) (Hietala *et al*. 2013).

Ash dieback symptoms (Figure 1) can be observed in shoots, twigs, branches and leaves and include lesions, dieback and necrosis. The ash crown is a good indicator of the presence of the disease, although this part of the tree is also affected by cankers. The disease can also induce premature abscission of foliage and wood discolouration (Johansson *et al*. 2010). Nevertheless, the absence of symptoms does not indicate good health of a tree, as infections by *Hymenoscyphus pseudoalbidus* can be present without apparent symptoms (Bakys *et al*.2009).

The first case of current ash dieback was observed in the early 1990s in North-Eastern Poland, but the causal agent was unknown at this time (Pautasso *et al*. 2012). It was several years later when *Hymenoscyphus pseudoalbidus* was identified as the pathogen. This Ascomycota is an invasive species, probably introduced from China or Japan where it is associated with different species of *Fraxinus* sp. and causes mild or no disease symptom (Zhao *et al*. 2012).

Nowadays, ash dieback is the main treat to biodiversity associated with *F. excelsior*. Common ash is widely distributed and has a keystone role in flood pain ecosystems. Indeed, a decreasing ash population will have both direct and indirect ecological effects on ecosystem services and biodiversity in woodlands, field margins, hedgerows and trees in agro-ecosystems (Pautasso *et al*. 2013).

Invasive species can have a large impact on native species, including hybridisation, introgression, niche displacement, competitive exclusion and extinction (Mooney and Cleland 2001). For a tree, the impact of invasive fungi can be multiple. One example is the fungus *Ophiostoma novo-ulmi* which has caused massive eradication of mature elm trees (Brasier 1991). The effects can also occur by changing the natural ecosystem of the tree. The purpose of this study is the observation of these phenomena.

Hymenoscyphus pseudoalbidus is an aggressive pathogen, gaining access to the host plant through the foliage, but its impacts on the endophytic fungi in Oleaceae are unclear. Endophytic fungi are mutualists that live within a plant for at least part of the life cycle without causing apparent disease. The relationship between an endophyte and a plant can be mutualistic, mainly through the action of mycotoxins, for example alkaloids in infected grasses, which protect the host plant from herbivory (Redlin and Carri, 1996).

Endophytes can have impacts on plants at various different levels: morphological, physiological and developmental. Their presence can give the plant a competitive advantage in stressful environments (Faeth, 2002), for example, influencing growth. Little is known of how a pathogen may impact on the endophyte-tree relationship (Redlin and Carri, 1996). Investigating interactions between endophytic fungi and *H. pseudoalbidus* may result in novel methods to protect ash against the pathogen.

Hymenoscyphus albidus is a non-pathogenic species that infects ash in Europe. This saprotroph is wide spread in Europe and the fruiting bodies can frequently be found in leaf litter where it acts as a primary decomposer (McKinney *et al. 2012)*. Although *H. albidus* infects ash foliage, it does not cause dieback or any other symptoms, although Zhao *et al*. 2012 suggested it was responsible for dieback. *H. albidus* and *H. pseudoalbidus* are morphologically similar and it is hard to distinguish between the two species. However with molecular analyses it is relatively simple to determine, as differences are found at, for example, the calmodulin[1] locus (Queloz *et al*. 2011).

McKinney *et al*. (2012) demonstrated than the presence of *Hymenoscyphus pseudoalbidus* leads to the competitive exclusion of *H. albidus* from its natural ecological niche. This phenomenon provoked the extinction of *H. albidus* in Denmark. Little is known about competitive exclusion when fungi are involved. Indeed, few cases have been reported where the mechanisms were well understood. One example of the phenomenon with fungi is again with Dutch elm disease, where the less-aggressive pathogen *Ophiostoma ulmi* was replaced by the more aggressive *Ophiostoma novo-ulmi*; but for *Hymenoscyphus* sp. the competitive exclusion process is still vague. The large distribution of *H. pseudoalbidus* could also be due to its rapid rate of spread in comparison to *H.albidus* (McKinney *et* al. 2012).

Studying both species in the same context can be interesting due to these apparently small differences and may help to reveal why *H. pseudoalbidus* is pathogenic.

[1]Calmodulin : is a calcium-binding messenger protein expressed in all eukaryotic cells

In a previous study, (Madigan 2014) the potential role of other Oleaceae (*Ligustrum vulgare* and *Forsythia x intermedia)* in the spread of *H. pseudoalbidus* was examined. At the same time, the diversity of endophytic fungal communities associated with several hardy woody Oleaceae (*Forsythia, Fraxinus, Ligustrum* and *Syringa*) was examined. The family Oleaceae includes some 600 species in 25 genera; many species are of particular economic importance and others are used extensively for ornamental purposes. Despite this importance, little was known of the endophytic fungal community in Oleaceae. It was shown that *H. pseudoalbidus* did not behave as a pathogen on *Ligustrum vulgare* or *Forsythia x intermedia*, as it did not colonize wounds on young shoots. In addition, Madigan (2014) demonstrated that the hardy Oleaceae tested contained high numbers of endophytic fungi. Interactions between the pathogen *Hymenoscyphus pseudoalbidus* and these endophytic species were not tested, however. It is possible that endophytes may provide the key to protecting *Fraxinus excelsior* and *F. angustifolia* against the ash dieback pathogen.

The aim of the work described in this thesis, therefore, was to determine interactions occurring in vitro between endophytic fungi isolated from *Fraxinus excelsior*, *Forsythia* x *intermedia* 'Lynwood', *Ligustrum vulgare* and *Syringa emodi* and the two *Hymenoscyphus* species, *H. pseudoalbidus* and *H. albidus* on malt extract agar, compared with malt extract agar containing extract of ash foliage.

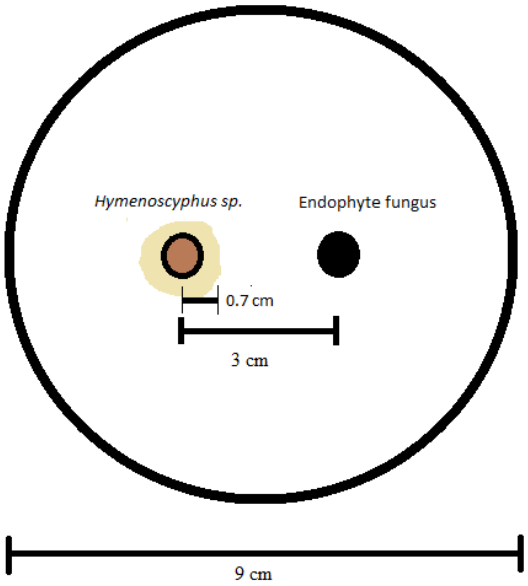


Figure 2: Schematic representation showing the placement of Hymenoscyphus *sp. and the test endophytic fungus in a Petri dish.*

II] Materials and methods

1) Choice of isolates

Three *Hymenoscyphus pseudoalbidus* isolates were used in the interaction tests, two from Austria: STL 1/6 (Austria, Styria, St.Lorenenob Murau, 2010) and NWE/1/2/H1 (Austria, Vienna, Neuwaldegg, 2008), their GenBank accession numbers are, respectively KC529349 and KC529351. The third isolate, L19, was from Central Lithuania.

In addition, two *Hymenoscyphus albidus* isolates were chosen, Car/5 (GenBank number: KC509944) and Car/6 (GenBank number: KC509945) both from France Brittany, (Carnac 2012).

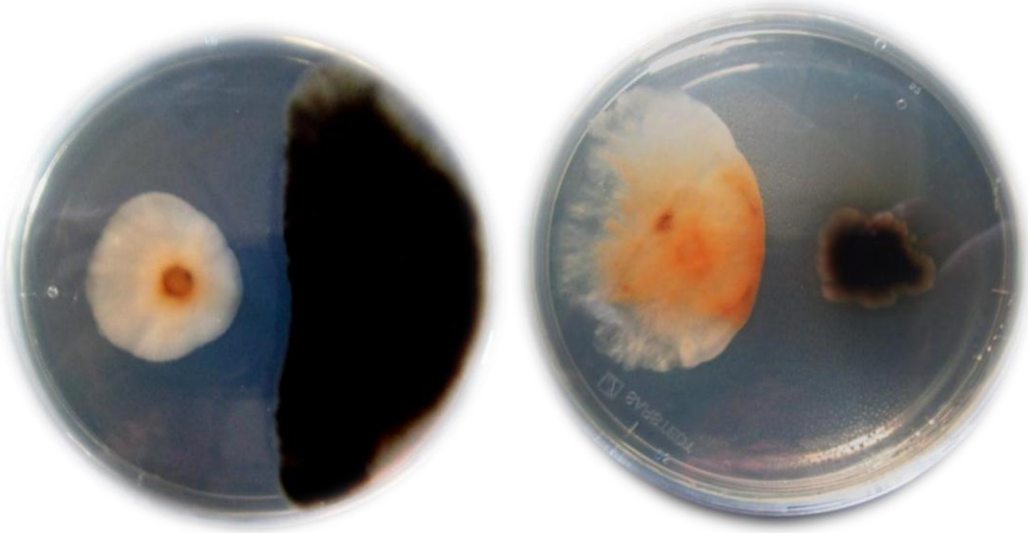
A large number of foliar endophytic fungi from hardy Oleaceae from a previous study in the same laboratory (Madigan, 2014) in which the isolates were sequenced. Thirty four isolates were selected for the interactions work, based on the species isolated from the foliage of four species of Oleaceae growing in the Cruickshank Botanic Gardens, Aberdeen, UK:

- *Ligustrum ovalifolium* also known as oval-leaved privet (cultures named 'PS')
-*Ligustrum vulgare aureovariegatum* commonly known as wild privet 'Aureovariegatum' (cultures named 'PC')
- *Forsythia x intermedia* 'Lynwood' (cultures named 'F' and 'FZ')
- *Syringa emodi*or Himalayan lilac (cultures named 'S').

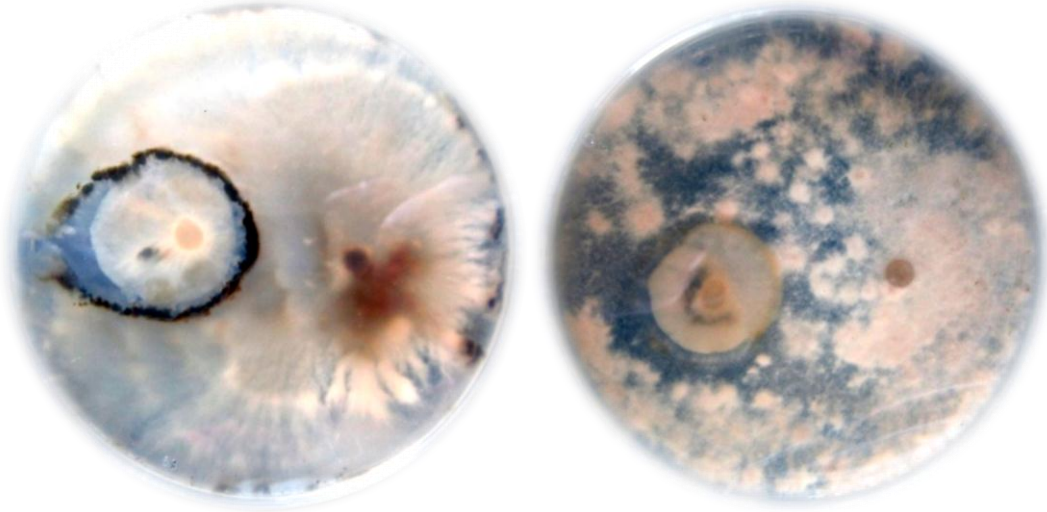
2) Subculture and dual culture interaction

Each isolate was subcultured into two 90 mm diam. Petri dishes containing 2% malt extract agar (MEA) and incubated at 22ºC to check for viability.

Dual cultures were prepared by placing inoculum of isolates at a distance of 3cm from each other using a template (Figure 2). A 90 mm diameter cork borer was used to cut inoculum from the actively growing cultures of the test isolates. As the *Hymenoscyphus* sp. grew more slowly than the endophytic species, it was necessary to inoculate the *Hymenoscyphus* sp. isolates several days before the other species (Rees, 2014).This time period varied according to the *Hymenoscyphus* sp. isolate. Hence, endophytic species were placed 30mm from the leading edge of the *Hymenoscyphus* sp., after the *H. albidus* or *H. pseudoalbidus* colony had a radius of 7 mm (Figure 2).



(a) : Inhibition (b): Inhibition of *Hymenoscyphus* sp.



(c): Barrage (d): Over growing

Figure 3: Different interaction typesobserved between Hymenoscyphus *sp. (left on each image) against the endophyte fungus (right).*

Dual cultures were also prepared using two *Hymenoscyphus pseudoalbidus* isolates (NWE/1/2/H1 and L19) in ash leaf malt extract agar (AMEA: MEA amended with 50 g fresh *F. excelsior* leaflets; leaflets were removed after autoclaving (Kirisits *et al*. 2013).

Interactions between each isolate of *H. pseudoalbidus*, between isolates of *H. pseudoalbidus* and *H. albidus*, and between isolates of *H. albidus.* For these tests, *H. albidus* was sub-cultured first and the *H. albidus* added when the *H. pseudoalbidus* colony had reached a diameter of 0.7cm.

Interactions in dual cultures were examined at different times of growth and classified into four qualitative categories; two intermediate stages were also used when it was not possible to rank interactions in the four categories (Figure 3):

- I: Inhibition (Figure 3(a)): a clear inhibition zone free of hyphae formed between the two mycelia. This category included inhibition of the endophyte due to the presence of the *Hymenoscyphus* sp. or when both fungi were inhibited by each other.
- I/: Inhibition of *Hymenoscyphus* sp. (Fig. 3(b)): the endophyte inhibited growth of *Hymenoscyphus* sp.
- B: Barrage (Fig. 3(c)): both fungi ceased growth on physical contact; mycelia of the two isolates then formed a substantial mass of aerial mycelium, sometimes with intermingling.
- G: Over growth: (Fig. 3(d)). Hyphae of the two tests isolates intermingled in the zone of contact. Both fungi grew without visibly inhibiting one another.
- Nothing yet (N): At the time of observation, insufficient growth had occurred for accurate assessment of interaction outcomes.
- No identify (inc): early in the incubation period, some interactions could not be clearly assigned one of the above categories.

Table 1: Growth rates for *Hymenoscyphus* sp. on MEA or AMEA.

Isolate identification	Time speed on MEA (in cm/day)	Time speed on AMEA (in cm/day)
Hymenoscyphus pseudoalbidus		
NWE/1/2/H1	0.14	0.14
STL 1/6	0.05	
L19	0.037	0.14
Mean	0.08(± 0.05)	0.14
Hymenoscyphus albidus		
CAR/6	0.05	
CAR/5	0.025	
Mean	0.0375(± 0.0125)	

Figure 4. Curves showing the number of interaction (in %) for each category, between NWE/1/2H1 and the endophytes, in function of the time (in days)

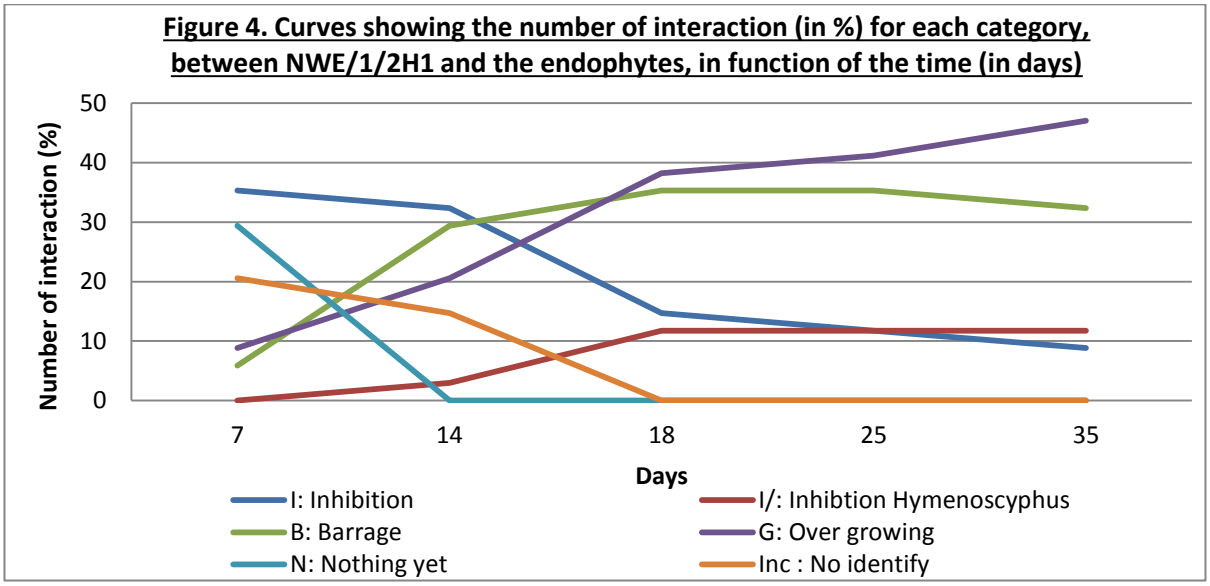
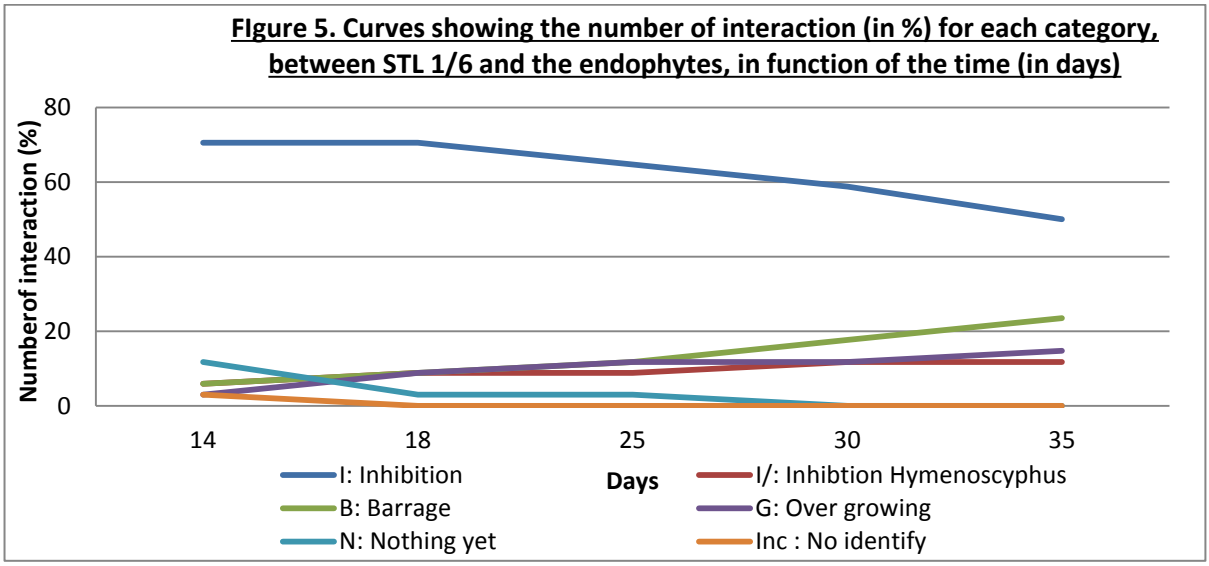


FIgure 5. Curves showing the number of interaction (in %) for each category, between STL 1/6 and the endophytes, in function of the time (in days)



III] Results

1) Time of growth and AMEA media

Table 1 shows the speed of growth of *Hymenoscyphus pseudoalbidus* and *H. albidus* on the two different media used. The isolates all had different growth rates. For *H. pseudoalbidus* growth rates varied between 0.14 (NWE/1/2/H1 on MEA) to 0.037cm/day (L19 on MEA). *H. albidus* isolates were slower in growth, with a mean of 0.0375(± 0.0125)cm/day.

Growth of the isolates on the two media also differed. Isolate NWE/1/2/H1 had the same growth rates on both MEA and AMEA. *H. pseudoalbidus* isolate L19 was slow growing in MEA (0.037cm/day) but have the same growth rate as NWE/1/2/H1 on AMEA.

The colour of *H. pseudoalbidus* colonies differed on the two media: on MEA the colour was white cream, whereas on AMEA it was brown orange.

2) Dual interactions

a) Development with time

Interactions between *Hymenoscyphus* sp. and the endophytic fungi initially showed a high percentage of inhibition against the endophyte, which varied in numbers between isolates, from 35% for NWE/1/2/H1 (Figure 4) to 80% for Car/6 (Figure 7). For isolates L19 (Figure 6) and CAR/5 (Figure 8), no interactions were observed for 35% and 56% of dual cultures, in the first 7 days. Percentage of inhibition slowly decreased with time, to 9% for NWE/1/2/H1 (Figure 4), 50% and 53% for STL 1/6 (Figure 5) and L19 (Figure 6) and 41% for CAR/6 (Figure 7)

With time, inhibition, barrage formation and over-growth against of *Hymenoscyphus* sp., increased for all isolates (Figure 4), although over variable time periods for the different endophytic species.

The number of *Hymenoscyphus* inhibited is twelve % and represent four fungi on 32 endophytes and to reach this last stage takes different times for each isolate: 18 days for NWE1/2/H1, 30 days for STL 1/6, 14 days for L19, and 30 days for CAR/6. For the isolate CAR/5 this percentage is only 9% due to the absence of data for some endophyte-isolate interactions.

The number of over-growth interactions increased with time for each *Hymenoscyphus* isolate. Nine % of endophytes had overgrown isolate NWE/1/2/H1 in 7 days, although after 35 days, 47% had over-grown the pathogen. The changes mostly occurred in the first 18 days of interaction. Over-growth of isolates, STL 1/6 and L19 also increased, although at 35 days 15% and 12% of endophytic species had overgrown them, respectively. For *H. albidus* isolates overgrowth was 28% for CAR/6 and 24 % for CAR/5 at respectively 35 days and 25 days.

Figure 6. Curves showing the number of interaction (in %) for each category, between L19 and the endophytes, in function of the time (in days)

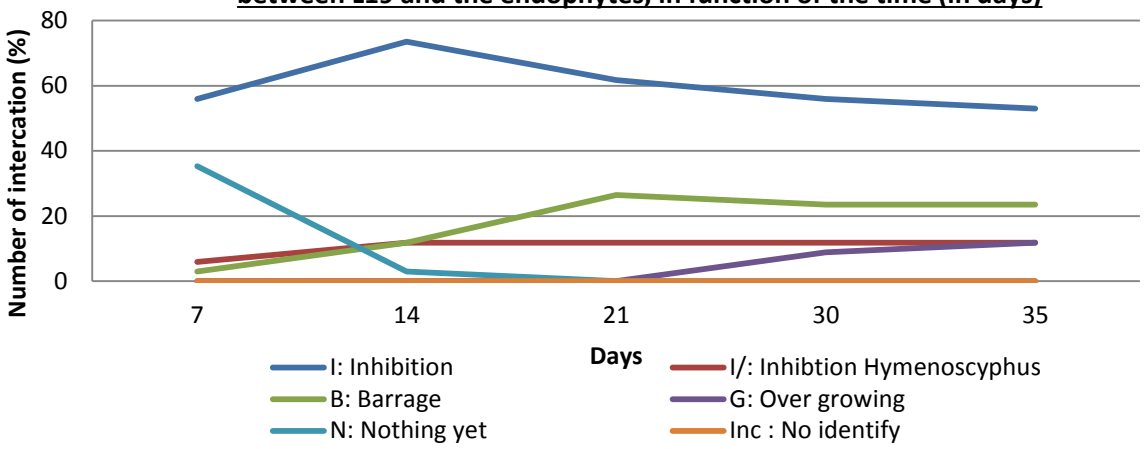


Figure 7. Curves showing the number of interaction (in %) for each category, between CAR/6 and the endophytes, in function of the time (in days)

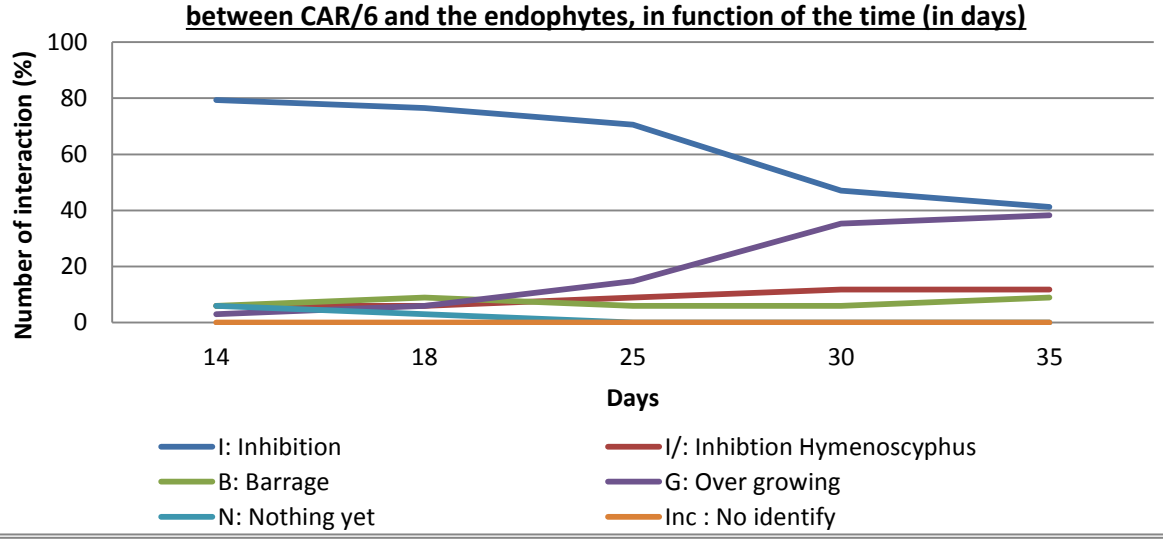
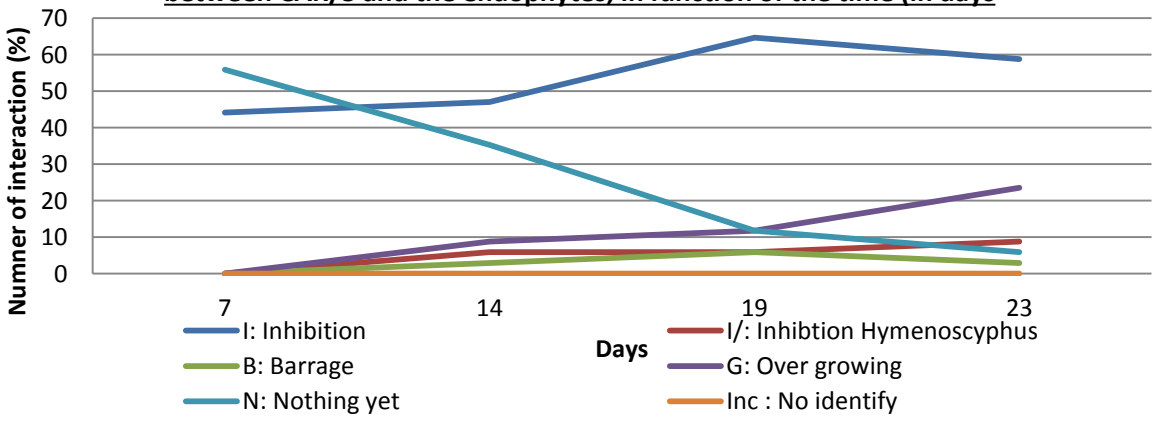


Figure 8. Curves showing the number of interaction (in %) for each category, between CAR/5 and the endophytes, in function of the time (in days



The number of barrage interactions also increased with time for each *H. pseudoalbidus* isolate. For NWE/1/2/H1 and L19 barrage formation increased greatly during the 20 first days to reach 35% and 26 %, respectively, then remained constant or slowly decreased. With STL 1/6 numbers of barrages formed increased throughout the incubation period to 24%. *H. albidus* CAR/6 and CAR/5 had lower percentage barrage formation, varying between 6 and 9%, and 3 and 6%, respectively.

For current reporting purposes, observations of interactions between *H. pseudoalbidus* and the endophytic fungi on AMEA was only possible one week after inoculation. Both *H. pseudoalbidus* isolates required more time to grow compared with rates on MEA. Interactions at this time were only in two categories: “nothing yet” for 81% (NWE/1/2/H1) and 91% (L19) (appendix 1); the others were classed as “over-growth”, with one exception in the NEW/1/2/H1 confrontation with *E. nigrum* (PC7) where inhibition was observed.

b) Changes between interaction categories

To understand these results a link must be established between each interaction category. Table 2 summarises changes in interaction category with time (Appendix 2) Some fungi were in the categories of inhibition, barrage formation or over-growth throughout the incubation period, as for example, with *Botryosphaeria sarmentorum* against each *Hymenoscyphus* isolate (inhibition of the endophyte only), *Phoma exigua* against STL1/6 (only barrage formation), *Botryotinia fuckeliana* against NWE/1/2/H1 (only over-growth), *Plenodomus influorescens* against each isolate (inhibition of *Hymenoscyphus* sp.). Changes in interactions varied (Table 2):

- Inhibition-Barrage: after a certain time of displaying an inhibition interaction, barrage formation is observed and the zone free of hyphae is no longer present. One example was the interaction between *Glomerella acutata* and NWE/1/2/H1 and STL 1/6, where after a time of inhibition barrage formation occurred.

- Inhibition-Over-growth: this interaction started with inhibition, which after time was followed by over-growth of the fungi. This interaction was observed, for example with *Epicoccum nigrum* against NWE1/2/H1 in which inhibition of the endophyte was followed over-growth by both species.

- Inhibition-Barrage-Over-growth: this type of interaction was the most complex and started with inhibition of the endophyte, followed by the formation of barrage by the endophyte and then further growth of the endophyte over the *Hymenoscyphus* sp. isolate. This change was observed commonly, for example, in the *Diaporthe viticola*– NWE/1/2/H1 and L19 interactions, or *Phomopsis* sp. against NWE/1/2/H1 or CAR/6.

Table 2: Changes in interaction type with time.I: Constant inhibition, I/: Constant inhibition of *Hymenoscyphus* sp. G: Constant over-growth, B: Constant barrage, I-G: Inhibition to Over-growth, I-B: Inhibition to Barrage, I-B-G: Inhibition-Barrage-Over-growth, B-G:Barrage-Over-growth.

Endophyte		**NWE/1/2/H1**	**L19**	**STL1/6**	**CAR/6**	**CAR/5**
Alternaria alternata	S1	B-G	I-B	B-G	B-G	I-G
Aureobasidium pullulans	FZ41	I-B-G	I	I	I	I
Botryosphaeria sarmentorum	F27	I-B-G	I-B-G	I-G	I-G	I
B. sarmentorum	F27x	I	I	I	I	N
Botryotinia fuckeliana	S34	G	I-B	I-G	I-B-G	I-G
B.fuckeliana	FZ22	G	B-G	I-G	G	G
Cladosporium cladosporioides	FZ32	I-B-G	I	I	I	I
C. cladosporioides	PS24	I	I	I	I	I
Cadophoraluteo-olivacea	PS30	I	I	I	I	I
Colletotrichum rhombiforme	FZ12	I-B	I-G	G	I	I
Cytospora sp.	F42	I-G	I	I	I-G	I
Diaporthe viticola	PS12	B	I-B	I-B	B	I-G
D. viticola	S5	I-B-G	I-B-G	I	I-G	B-G
D. viticola	PC18	I-G	B	I-B	I-G	I
Epicoccum nigrum	FZ18	I-G	I	I	I-G	I
E. nigrum	PC7	I-G	I	I	I	I-G
E. nigrum	S26	I-G	I	I	I-G	I
Fusarium lateritium	F17	I/	I/	I/	I/	I/
Glomerella cingulata	FZ33	I/	I/	I/	I/	I/
Glomerella acutata	F60	I-B	I	I-B	I-G	I
Lewia infectoria	PC8	B-G	I-B	I-B	I-G	I-G
Mycosphaerella coacervata	PC28	I-B	I	I	I-B	I
Phoma exigua	PC13	I-B	I	B	I	I
P. exigua	S38	I-G	I-B	I-B	I	I
Phoma macrostoma	PS10	B	I	I	I	I
P.macrostoma	F29	I-B	I	I	I	I
P. macrostoma	S50	I-B	I	I	I-G	B
P. macrostoma	PC21	I-B	I	I	I	I
Phoma viburnicola	F39	I-B	I-B	I	I	I
P. viburnicola	PC5	I	I	I	I	I
Phomopsi ssp.	F34	G	I-B	I-B	I-B-G	G
Phomopsis sp.	PS20	I-B-G	B	I-B	I-B	I
Plenodomus influorescens	F23	I/	I/	I/	I/	I/
Sordariomycetes sp.	PS31	I/	I/	I/	I/	N

- Barrage-Over-growth: a similar change to Inhibition-Barrage-Over-growth, but lacking the initial as inhibition phase. This type of change was found with *Alternaria alternate* against NWE/1/2/H1, STL 1/6 or CAR/6.

No species-specific interactions were observed, although differences between isolates of given species did occur. For example, with *Lewia infectoria,* both isolates of *H. albidus* had the Inhibition-Over-growth interaction change, but all *H. pseudoalbidus* isolates reacted differently: NWE/1/2/H1 showed a Barrage-Over-growth change, but an Inhibition-Barrage change with isolates L19 and STL 1/6.

c) Identical endophytes isolated from different trees

The choice to use the complete endophyte community from different hardy Oleaceae (*Ligustrum ovalifolium, Ligustrum vulgare aureovariegatum*, *Forsythia x intermedia*, *Syringa emodi*) enabled comparison of the interactions of fungal endophytic communities against the pathogen. It is the case for B*otryosphaeria sarmentorum*, *Botryotinia fuckeliana*, *Cladosporium cladosporioides, Diaporthe viticola, Epicoccum nigrum, Phoma exigua, Phoma macrostoma, Phoma viburnicola, Phomopsis* sp which been isolated on each trees.

Generally it was rare to obtain precisely the same results for confrontations against each *Hymenoscyphus* sp. but there was a trend in how the fungi reacted against a same isolate. For example, the different isolates of *E. nigrum* reacted similarly in dual culture with the *H. pseudoalbidus* isolates: inhibition and over-growth against NWE/1/2/H1, compared with inhibition against L19 and STL 1/6. Against CAR/6, however, the difference isolates of *E. nigrum* gave different responses: constant inhibition for the endophyte from *Ligustrum ovalifolium*, in contrast to the Forsythia x *intermedia* and *Syringa emodi* isolates which began with inhibition but changed to over-growth with time; CAR/5 reacted in the opposite way: constant inhibition for the endophyte from Forsythia x *intermedia* and *Syringa emodi,* in contrast to the endophyte from *Ligustrum ovalifolium*.

This result was not an exception. *Phoma macrostoma,* for example, which was isolated from the four woody plant species, reacted against NWE/1/2/H1 with an inhibition response, changing to barrage formation, except for the isolate coming from *Ligustrum ovalifolium* where barrage formation occurred quickly. With the *P. macrostoma* isolates from *S. emodi*, the confrontation began with inhibition followed by over-growth. It is likely that the response was the same for each *P. macrostoma* isolate and each host but at different rates during incubation. The rapid barrage formation with the *L. ovalifolium* was probably due to a rapid inhibition phase, which was not seen because of the timing of observations.

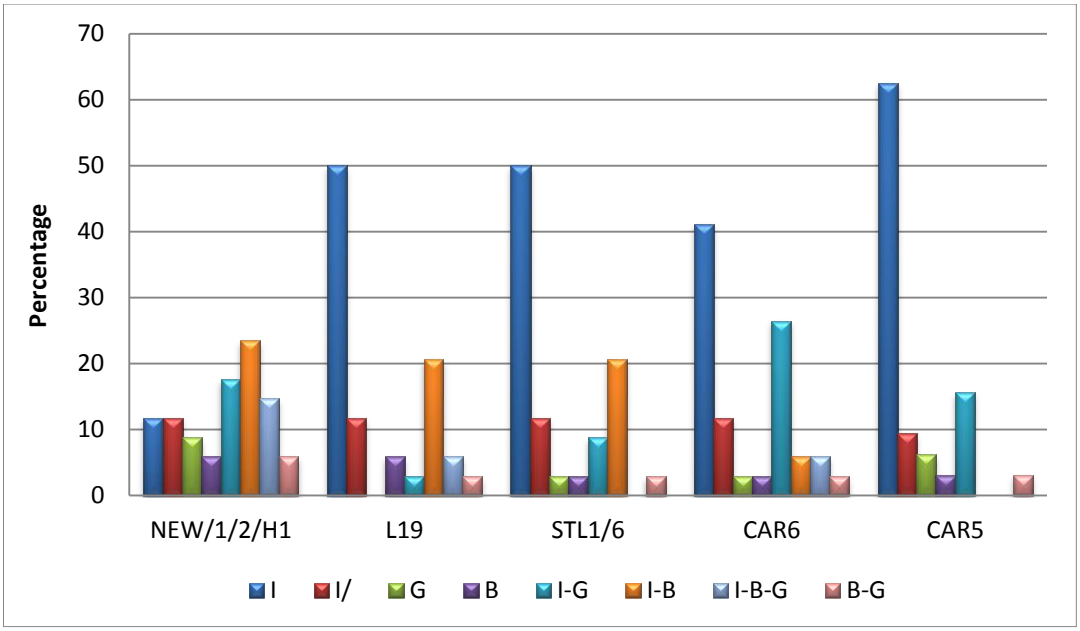


Figure 9: Bart chart showing the numbers of each type of interaction evolution by percentage. I: Constant inhibition, I/: Constant inhibition of *Hymenoscyphus* sp. G: Constant over-growth, B: Constant barrage, I-G: Inhibition to Over-growth, I-B: Inhibition to Barrage, I-B-G: Inhibition-Barrage-Over-growth, B-G:Barrage-Over-growth.

Table 3: interaction between the different isolates in function of the time.

		20 days	***32 days***	***39 days***	***41 days***
L19	NWE/1/2/H1	N	G	G	G
L19	L19	N	G	G	G
L19	STL 1/6	N	G	G	G
L19	CAR/6	G	G	G	G
L19	CAR/5	N	N	N	N
STL 1/6	CAR/6	I (C on S)	I (C on S)	G	G
STL 1/6	CAR/5	N	N	N	N
STL 1/6	STL 1/6	N	G	G	G
STL 1/6	NWE/1/2/H1	N	G	G	G
NWE/1/2/H1	NWE/1/2/H1	G	G	G	G
NWE/1/2/H1	CAR/6	N	G	G	G
NWE/1/2/H1	CAR/5	N	N	N	G
CAR/6	CAR/6	N	I	I	I
CAR/6	CAR/5	N	N	N	N
CAR/5	CAR/5	N	N	N	N

Some other results are harder to explain, such as *Botryosphaeria sarmentorum* for which two isolates from the same tree (*Fraxinus excelsior*) gave different results. *Botryosphaeria sarmentorum* F27 showed an Inhibition-Barrage-Over-growth response with *H. pseudoalbidus* NWE/1/2/H1 and L19, but Inhibition-Over-growth with *H. pseudoalbidus* STL 1/6 and *H. albidus* CAR/6); *B. sarmentorum* F27x, in contrast, inhibited *H. pseudoalbidus* and *H. albidus*.

d) Difference between H. pseudoalbidus and H. albidus

Changes in interactions between *H. pseudoalbidus* and *H. albidus* are shown in Figure 9. *H. pseudoalbidus* NWE/1/2/H1 showed all types of interaction changes possible with time, distributed between 5 and 23 %. The biggest change was from inhibition to barrage formation. The two other isolates of *H. pseudoalbidus* were more likely to inhibit the endophyte, and 50% of these interactions did not change with time. The change from inhibition to barrage formation occurred in 20% of the interactions. The *H. albidus* isolates, CAR/6 and CAR/5, generally inhibited the endophytes, with percentages of inhibition between 42 and 62% but, in contrast to *H. pseudoalbidus*, the second most important type of interaction change with *H. albidus* was inhibition followed by over-growth.

e) Hymenoscyphus *sp. against* Hymenoscyphus *sp.*

Table 3 shows the interaction results between the different isolates of *Hymenoscyphus* species. Due to the slow growth rates of the *Hymenoscyphus* sp. no interactions were recorded the 7 days of incubation. Over-growth was observed for the first time after 20 days of incubation.

However two confrontations are not the same. The interaction STL 1/6 against CAR/6 and the interaction of CAR/6 against itself showed an inhibition interaction. *H. albidus* CAR/6 appeared to inhibit STL 1/6. Later in the interaction, this inhibition was followed by over-growth.

IV] Discussion

1) Accuracy of the results

Dual confrontations were performed with 3 isolates of *Hymenoscyphus pseudoalbidus* and 2 of *H. albidus*, against 34 endophytic fungi previously isolated from hardy woody Oleaceae (Madigan 2014). Longer experimental times are required to ascertain the true nature and outcomes of these interspecific interactions. For example, due to the slow growth rate of *H. albidus* CAR/5, it was difficult to confirm the interaction outcomes compared with isolate CAR/6 which grew more rapidly.

Currently, interpretation of the results from the substrate amended with ash foliage is limited to the first week of the confrontation, as a result of the late flushing time of ash in the north of Britain. This limited time only allowed comparison of substrate amended with foliage and MEA after one week of incubation.

These results have to be analysed in terms of appropriate conditions for growth without the external impact of environmental factors. Laboratory conditions eliminate the impact of changing temperatures, changing luminosity, and the presence of other organism within the inter-specific interactions. These factors may have negative or positive impacts on the endophytic fungi or be beneficial to the protection of the tree against the pathogen. U ' Ren *et al*. (2012) showed that climatic patterns, geographic separation, host type and host lineage can have an effect on the composition of endophytic fungal communities.

2) Growth rates and interactions of *Hymenoscyphus* sp.

The slow rate of *H. albidus* isolate CAR/5 meant that the dual culture interaction tests were started later than for the other isolate. This problem led to a lack of the final data on this isolate. Most interaction tests were examined for the last time after 35 days.

Hymenoscyphus pseudoalbidus was much faster growing than the non-pathogenic species *H. albidus*, as previously demonstrated by Kirisits *et al.* (2012), although growth rates were more rapid than found previously. Kirisits *et al.* (2012) also proved that both species grew more rapidly when MEA was amended with ash foliage, as confirmed in this work, particularly for *H. pseudoalbidus*. Clearly, more rapid fungal growth is useful in time-limited experiments.

The interaction between *H. pseudoalbidus* and *H. albidus* was a major question previously unanswered. The non-pathogenic *H. albidus* is a common endophytic fungus on ash in Europe, important in the decomposition of leaf litter. McKinney *et al*. (2012) hypothesised the possible extinction of *H. albidus* due to competitive exclusion by *H. pseudoalbidus*. This study hypothesized that exclusion may be due to the faster growth rate of *H. pseudoalbidus*, which was supported by simple observation in this work. Further support was provided by the observations of dual cultures between the two *Hymenoscyphus* sp. As the confrontation was performed in two different ways, firstly when *H. albidus* was inoculated initially and then when *H. pseudoalbidus* had already grown to some extent¸ the growth rate was clearly of little significance, as the interaction outcomes were the same: over-growth.

3) Dual interaction

a) Different interaction categories

Overall, inhibition of the endophyte was the most common interaction observed, although it decreased with time. Barrage formation was the second most common interaction between the pathogenic fungus and the endophytes. In contrast the non-pathogenic fungus showed more over-growth interactions with the endophytes, whilst numbers of barrage formations remained low. The NWE/1/2/H1 interactions were an exception to these generalisations, with barrage formation occurring more commonly initially and, as time progressed, over-growth became more predominant.

The dual interactions were classified into four categories: inhibition of the endophyte by *Hymenoscyphus*, inhibition of *Hymenoscyphus* by the endophyte, barrage formation and over-growth. All interactions observed could be placed in one of these categories, justifying this choice.

A general observation was the high proportions of dual cultures showing an inhibition interaction (between 35% and 80%) in the early stage of co-culture, although these proportions decreased with time. There was clearly a succession in the relationships between the species in co-culture. Inhibition can result from strong competition for nutrients present in the growth substrate, when the fungus showing the strongest growth prevents the other species growing (Sussman, 1968). This type of interaction can be simply compared in a medium with high nutritional content, which is not limiting, such as MEA. The AMEA substrate used here was also a good indicator for this type of interaction due to the additional nutrients from the ash foliage. Inhibition can also result from the production of growth inhibitors into the media by one or both of the interacting species. The production of antibiotics is widespread in fungi (Smith and Berry, 1978): many species produce secondary metabolites which can inhibit or kill the antagonist fungi at some distance (Sussman, 1968).

Whilst growth of most of the endophytic fungi tested here was inhibited in the presence of *Hymenoscyphus* sp.*, Plenodomus influorescens, Glomerella cingulata, Fusarium lateritium* and the Sordariomycete species produced secondary metabolites which diffused through the substrate and reduced the growth of *Hymenoscyphus*. In some interactions, it is possible that the metabolites caused lysis of *Hymenoscyphus,* as recorded with other fungi by the use of diffusion of cell wall degrading enzymes, such as chitinases and/or glucanases (Zeilinger and Mukherjee, 2013). This result can also be the result of a strong competition for the nutriment in the substrate between the two fungi resulting in inhibition of the pathogen.

This result could be interesting for work on preventing ash dieback through biological control. Nevertheless the use of mycoparasitism, where one fungus attacks another, it is not well understood and the consequence of using such a system requires thorough investigation (Redlin and Carris, 1996). The four fungi inhibiting growth of *Hymenoscyphus* in the present work are more or less well known:

- *Plenodomus influorescens*: little is known about this fungus, which was isolated from *Forsythia x intermedia*
- *Glomerella cingulata*: also isolated from *Forsythia x intermedia*. This fungus is widely known as a pathogen and distributed globally, infecting various plants, causing anthracnose (Marumoto and Miyazawa, 2009). It has been investigated in biotransformation and biocatalysis to produce bioactive terpenoids[2] (Miyazawa and Shimizu, 2011)
- *Fusarium lateritium*: isolated here from *Forsythia x intermedia*. This species is also a globally distributed plant pathogen. It was recently reported as the causal agent of nut gray necrosis on hazelnut (Vitale *et al*. 2011).
- *Sordariomycetes* sp: from *Syringa emodi* [GenBank accession JQ760920.1]. Clearly, little is known of this species, as it is not completely characterised and named. Sequences were deposited in GenBank by U'Ren *et al*. (2012).

Over-growth was the second-most common type of fungus-fungus interaction recorded in the present work. With greater incubation time this type of interaction was more common for all isolates. With isolates of *H. albidus* and *H. pseudoalbidus* NWE/1/2/H1 this interaction was the second most common at the end of the recording period. This type of interaction may simply be due to a non-damaging interaction between the endophyte and *Hymenoscyphus.* There are sufficient nutrients for coexistence and any antibiotics or cell wall lysing enzymes are not produced in sufficient quantities to affect growth. Alternatively, one of the two species may be over-growing the colony of the other, in which case the contact phenomenon may include mechanisms of competition where the dominant fungus

[2]Terpenoids: class of naturally occurring organic chemicals similar to terpenes (organic compounds, produced by a variety of plants) with additional functional groups

produces cell wall degrading enzymes which lyse the hyphae of the second species. In the present work, classification in the over-growth category did not consider the possibility that lysis occurred, as the observation was simply that the endophyte grew over the interacting isolate.

Cell wall degradation can also explain the succession from inhibition to over-growth in the case of *Hymenoscyphus albidus.* Initially, inhibition occurred, such that *H. albidus* had a negative impact on the growth of the endophyte, but the endophytes in this type of relationship reacted by the lysis of *H. albidus* hyphae, resulting in over-growth.

Sussman (1968) also suggested how the over-growth of one fungus by another can result from the exploitation of the one organism for food. The over-growth interaction can also be a result of more rapid growth. A fast growing fungus may be a strong competitor and will grow despite the presence of the second fungus.

Barrage formation between fungi requires cytoplasmic contact via hyphal fusions (Esser and Meinhardt, 1984). In this work, barrage formation was observed most frequently as the final step of the interaction between an endophytic fungus and *Hymenoscyphus pseudoalbidus* following initial inhibition of endophyte growth. After the hyphal fusion has occurred, it is still possible that the fungi over-grow each other.

The reactions between the endophytes and *H. pseudoalbidus* indicated a possible impact of the introduction of an alien fungus into an ecosystem: competition can lead to niche displacement. All the interactions observed were the results of complex inter-specific relationships occurring at different levels, which in this case may impact on the tree disease.

b) Changes in interactions

An important result in this work, in terms of the ecology of these fungi was the change in different types of interactions with time. Inhibition was often followed by barrage formation or over-growth. Barrage formation was sometimes followed by over-growth, but no other type of succession was observed. Succession, where it occurred, was different for *H. pseudoalbidus* and *H. albidus.* Most often, succession with *H. pseudoalbidus* was inhibition followed by barrage formation (20%); for *H. albidus* the change was likely to be inhibition followed by over-growth (15 – 25% of interactions).

The succession inhibition-barrage formation-over-growth can be divided into different phases. Initially, inhibition is due to competition for resources, but as the nutrients diminish the fungi interact directly and barrage formation occurs if the hyphae fuse, or over-growth occurs, as described above.

c) Difference between source of tree

The endophytic communities from the four woody species had 9 endophytic fungi in common: *Botryosphaeria sarmentorum*, *Botryotinia fuckeliana*, *Cladosporium cladosporioides, Diaporthe viticola, Epicoccum nigrum, Phoma exigua, Phoma macrostoma, Phoma viburnicola* and *Phomopsis* sp. Nevertheless, even when these species were the same, the interactions varied when they were in the presence of a second fungus. Hence, it is only possible to define a tendency for a species to interact one way or another, and no hard rules can be made concerning what the outcome of culturing the species together will be. Genotype may also play a big role in interaction outcomes in plant tissues infected with the different species.

Conclusions

The purpose of this work was to determine the interactions between the pathogen *Hymenoscyphus pseudoalbidus* and the endophytic fungus *Hymenoscyphus albidus* with other endophytic fungi obtained from hardy woody Oleaceae species. These interactions were tested using endophytic fungal communities from four Oleaceae, and divided into four distinct categories: inhibition of the endophyte by *Hymenoscyphus*, inhibition of *Hymenoscyphus* by the endophytic fungus, barrage formation or over-growth. The interaction, however, was not fixed in time: the main tendency was an initial inhibition of the endophyte by *Hymenoscyphus* and then a decrease in this category whilst at the same time the other types of interaction increased. The interactions progressed from inhibition to barrage formation to over-growth. In dual cultures, four endophytic fungi inhibited both *H. pseudoalbidus* and the non-pathogenic *H. albidus.* The reasons for these variations remain vague and the mechanisms underlying these phenomena are unknown.

Comparison between the two *Hymenoscyphus* sp. suggested that both inhibited the endophytes early in the interaction. Differences between the two species were more obvious later in the experiment. The majority of confrontations with *H. albidus* produced over- growth interactions with a succession from inhibition to over-growth. In contrast, *H. pseudoalbidus* interacted more with barrage formation interactions, sometimes followed by over-growth. The reason these interactions occur in change in this manner are unknown, but further data from using the substrate amended with leaf foliage, and hence closer to that of the niche may help in understanding the results further. In dual cultures of *H. albidus* and *H. pseudoalbidus*, there was no exclusion of one species by the other, but general growth of both fungi in presence of each other.

Understanding these differences in response and the mechanisms controlling the interactions may help in protecting ash against dieback, and the further spread of *H. pseudoalbidus.*

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Appendix

Appendix 1 EMEA Result

			NEW/1/2/H1	L19
			7 days	7 days
Site : *Syringa emodi (site1)*	*Alternaria alternata*	S1	N	N
"S"	*Epicoccum nigrum*	S26	G	N
	Phoma macrostoma	S50	N	N
	Phoma exigua	S38	G	G
	Diaporthe viticola	S5	N	N
	Botryotinia fuckeliana	S34	G	G
Site : Zoo and CBG	*Aureobasidium pullulans*	FZ41	N	N
Forsythia x intermedia	*Cladosporium cladosporioides*	FZ32	N	N
	Phoma macrostoma	F29	N	N
	Botryosphaeria sarmentorum	F27	G	G
	Plenodomus influorescens	F23	N	N
	Glomerella cingulata	FZ33	N	N
	Epicoccum nigrum	FZ18	N	N
	Colletotrichum rhombiforme	FZ12	N	N
	Fusarium lateritium	F17	N	N
	Botryotinia fuckeliana	FZ22	G	G
	Glomerella acutata	F60	N	N
	Phomopsis sp	F34	G	N
	Phoma viburnicola	F39	N	N
	Cytospora sp.	F42	N	N
	Botryosphaeria sarmentorum	F27x	N	N
Site : Privet (Cruick)	*Phoma exigua*	PC13	G	G
Ligustrum vulgare aureovariegatum	*Epicoccum nigrum*	PC7	I	N
"PC"	*Phoma macrostoma*	PC21	N	N
	Lewia infectoria	PC8	N	N
	Mycosphaerella coacervata	PC28	N	N
	Phoma viburnicola	PC5	N	N
	Diaporthe viticola	PC18	N	N
Site : Privet (Side)	*Phomopsis sp.*	PS20	N	N
Syringa emodi	*Cladosporium cladosporioides*	PS24	N	N

"PS"	*Sordariomycetes sp.*	PS31	N	N
	Cadophora luteo-olivacea	PS30	N	N
	Diaporthe viticola	PS12	N	N
	Phoma macrostoma	PS10	N	N

Appendix 2 : interaction result

- NWE/1/2/H1 interaction Result :

			7 days	14 days	18 days	25 days	35 days
Site : *Syringaemodi (site1)*	*Alternaria ıata*	S1	B	B	B	G	G
"S"	*Epicoccum nigrum*	S26	I	G	G	G	G
	P. macrostoma	S50	I	I	B	B	B
	Phoma exigua	S38	I	I	G	G	G
	D. viticola	S5	I	B	B	B	G
	Botryotinia fuckeliana	S34	G	G	G	G	G
Site : Zoo and CBG	*Aureobasidium pullulans*	FZ41	I	I	B	B	G
Forsythia x intermedia	*Cladosporium cladosporioides*	FZ32	N	I	G	G	G
"FZ" and "F"	*P.macrostoma*	F29	I	B	B	B	B
	Botryosphaeria sarmentorum	F27	I	B	B	B	G
	Plenodomus influorescens	F23	N	I/	I/	I/	I/
	G. cingulata	FZ33	N	I/	I/	I/	I/
	E. nigrum	FZ18	I	G	G	G	G
	Colletotrichum rhombiforme	FZ12	I	B	B	B	B
	Fusarium lateritium	F17	N	I/	I/	I/	I/
	Botryotinia fuckeliana	FZ22	G	G	G	G	G
	Glomerella acutata	F60	I	I	I	B	B
	Phomopsis sp	F34	G	G	G	G	G
	Phoma viburnicola	F39	I	I	B	B	B
	Cytosporasp.	F42	I	G	G	G	G
	Botryosphaeria sarmentorum	F27x	I	I	I	I	I
Site : Privet (Cruick)	*Phoma exigua*	PC13	I	I	B	B	B

Ligustrumvulgareaureovariegatum	*E. nigrum*	PC7	Inc	I	G	G	G
"PC"	*Phoma macrostoma*	PC21	I	B	B	B	B
	Lewia infectoria	PC8	B	B	G	G	G
	Mycosphaerella coacervata	PC28	N	I	I	I	B
	Phoma viburnicola	PC5	I	I	I	I	I
	D. viticola	PC18	I	G	G	G	G
Site : Privet (Side)	*Phomopsis* sp.	PS20	I	B	G	G	G
Syringaemodi	*C. cladosporioides*	PS24	N	I	I	I	I
"PS"	*Sordariomycetes* sp.	PS31	N	I/	I/	I/	I/
	Cadophoraluteo-olivacea	PS30	N	I	I	I	I
	Diaporthe viticola	PS12	N	B	B	B	B
	Phoma macrostoma	PS10	N	B	B	B	B

- L19 interaction result

			7 days	14 days	21 days	30 days	35 days
Site : *Syringaemodi (site1)*	*Alternaria alternata*	S1	I	I	B	B	B
"S"	*Epicoccum nigrum*	S26	I	I	I	I	I
	Phoma macrostoma	S50	I	I	I	I	I
	Phoma exigua	S38	I	B	B	B	B
	Diaporthe viticola	S5	I	I	B	G	G
	Botryotinia fuckeliana	S34	I	B	B	B	B
Site : Zoo and CBG	*Aureobasidium pullulans*	FZ41	I	I	I	I	I
Forsythia x intermedia	*Cladosporium cladosporioides*	FZ32	N	I	I	I	I
"FZ" and "F"	*Phoma macrostoma*	F29	N	I	I	I	I
	Botryosphaeria sarmentorum	F27	I	I	B	G	G
	Plenodomus influorescens	F23	N	I/	I/	I/	I/

	Glomerella cingulata	FZ33	N	I/	I/	I/	I/
	Epicoccum nigrum	FZ18	I	I	I	I	I
	Colletotrichum rhombiforme	FZ12	N	I	I	I	G
	Fusarium lateritium	F17	I/	I/	I/	I/	I/
	Botryotinia fuckeliana	FZ22	B	B	B	G	G
	Glomerella acutata	F60	N	I	I	I	I
	Phomopsis sp	F34	N	I	I	I	B
	Phoma viburnicola	F39	N	I	I	B	B
	Cytospora sp.	F42	I	I	I	I	I
	Botryosphaeria sarmentorum	F27x	N	I	I	I	I
Site : Privet (Cruick)	*Phoma exigua*	PC13	I	I	I	I	I
Ligustrumvulgareaureovariegatum	*Epicoccum nigrum*	PC7	I	I	I	I	I
"PC"	*Phoma macrostoma*	PC21	I	I	I	I	I
	Lewia infectoria	PC8	I	I	B	B	B
	Mycosphaerella coacervata	PC28	N	N	I	I	I
	Phoma viburnicola	PC5	N	I	I	I	I
	Diaporthe viticola	PC18	N	B	B	B	B
Site : Privet (Side)	*Phomopsis sp.*	PS20	I	I	I	B	B
Syringaemodi	*Cladosporium cladosporioides*	PS24	I	I	I	I	I
"PS"	*Sordariomycetes sp.*	PS31	I/	I/	I/	I/	I/
	Cadophora luteo-olivacea	PS30	I	I	I	I	I
	Diaporthe viticola	PS12	I	I	B	B	B
	Phoma macrostoma	PS10	I	I	I	I	I

- STL 1/6 interaction result

			14 days	18 days	25 days	30 days
Site : *Syringaemodi (site1)*	*Alternaria alternata*	S1	B	B	B	B
"S"	*Epicoccum nigrum*	S26	I	I	I	I
	Phoma macrostoma	S50	I	I	I	I
	Phoma exigua	S38	I	I	I	I
	Diaporthe viticola	S5	I	I	I	I
	Botryotinia fuckeliana	S34	I	G	G	G
Site : Zoo and CBG	*Aureobasidium pullulans*	FZ41	I	I	I	I
Forsythia x intermedia	*Cladosporium cladosporioides*	FZ32	N	I	I	I
"FZ" and "F"	*Phoma macrostoma*	F29	I	I	I	I
	Botryosphaeria sarmentorum	F27	I	G	G	G
	Plenodomus influorescens	F23	N	N	N	I/
	Glomerella cingulata	FZ33	N	I/	I/	I/
	Epicoccum nigrum	FZ18	I	I	I	I
	Colletotrichum rhombiforme	FZ12	G	G	G	G
	Fusarium lateritium	F17	N	I/	I/	I/
	Botryotinia fuckeliana	FZ22	I	I	G	G
	Glomerella acutata	F60	I	I	I	I
	Phomopsis sp	F34	I	I	B	B
	Phoma viburnicola	F39	I	I	I	I
	Cytospora sp.	F42	I	I	I	I
	Botryosphaeria sarmentorum	F27x	I	I	I	I
Site : Privet (Cruick)	*Phoma exigua*	PC13	B	B	B	B
Ligustrumvulgareaureovariegatum	*Epicoccum nigrum*	PC7	Inc	I	I	I
"PC"	*Phoma macrostoma*	PC21	I	I	I	I
	Lewia infectoria	PC8	I	I	I	I
	Mycosphaerella coacervata	PC28	I	I	I	I
	Phoma viburnicola	PC5	I	I	I	I
	Diaporthe viticola	PC18	I	I	I	B
Site : Privet (Side)	*Phomopsis sp.*	PS20	I	I	I	B
Syringaemodi	*Cladosporium cladosporioides*	PS24	I	I	I	I
"PS"	*Sordariomycetes sp.*	PS31	I/	I/	I/	I/
	Cadophora luteo-olivacea	PS30	I	I	I	I

	Diaporthe viticola	PS12	I	B	B	B
	Phoma macrostoma	PS10	I	I	I	I

- CAR/6 interaction result

			14 days	18 days	25 days	30 days	35 days
Site : *Syringaemodi (site1)*	*Alternaria alternata*	S1	B	B	G	G	G
"S"	*Epicoccum nigrum*	S26	I	I	I	G	G
	Phoma macrostoma	S50	I	I	I	G	G
	Phoma exigua	S38	I	I	I	I	I
	Diaporthe viticola	S5	I	I	G	G	G
	Botryotinia fuckeliana	S34	I	B	G	G	G
Site : Zoo and CBG	*Aureobasidium pullulans*	FZ41	I	I	I	I	I
Forsythia x intermedia	*Cladosporium cladosporioides*	FZ32	I	I	I	I	I
"FZ" and "F"	*Phoma macrostoma*	F29	I	I	I	I	I
	Botryosphaeria sarmentorum	F27	I	I	I	G	G
	Plenodomus influorescens	F23	I/	I/	I/	I/	I/
	Glomerella cingulata	FZ33	I/	I/	I/	I/	I/
	Epicoccum nigrum	FZ18	I	I	I	G	G
	Colletotrichum rhombiforme	FZ12	I	I	I	I	I
	Fusarium lateritium	F17	N	N	I/	I/	I/
	Botryotinia fuckeliana	FZ22	G	G	G	G	G
	Glomerella acutata	F60	I	I	I	I	G
	Phomopsis sp	F34	I	I	B	G	G
	Phoma viburnicola	F39	I	I	I	I	I

	Cytospora sp.	F42	I	I	I	I	G
	Botryosphaeria sarmentorum	F27x	I	I	I	I	I
Site : Privet (Cruick)	*Phoma exigua*	PC13	I	I	I	I	I
Ligustrumvulgareaureovariegatum	*Epicoccum nigrum*	PC7	I	I	I	I	I
"PC"	*Phoma macrostoma*	PC21	I	I	I	I	I
	Lewia infectoria	PC8	I	I	I	G	G
	Mycosphaerella coacervata	PC28	N	I	I	I	B
	Phoma viburnicola	PC5	I	I	I	I	I
	Diaporthe viticola	PC18	I	G	G	G	G
Site : Privet (Side)	*Phomopsis sp.*	PS20	I	I	I	B	B
Syringaemodi	*Cladosporium cladosporioides*	PS24	I	I	I	I	I
"PS"	*Sordariomycetes sp.*	PS31	I	I	I	I/	I/
	Cadophora luteo-olivacea	PS30	I	I	I	I	I
	Diaporthe viticola	PS12	B	B	B	B	B
	Phoma macrostoma	PS10	I	I	I	I	I

- CAR/5 interaction result

			7 days	**14 days**	**19 days**	**23 days**
Site : *Syringaemodi (site1)*	*Alternaria alternata*	S1	I	I	G	G
"S"	*Epicoccum nigrum*	S26	I	I	I	I
	Phoma macrostoma	S50	I	I	I	I
	Phoma exigua	S38	I	I	I	I
	Diaporthe viticola	S5	I	G	G	G
	Botryotinia fuckeliana	S34	I	G	G	G
Site : Zoo and CBG	*Aureobasidium*	FZ4	I	I	I	I

	pullulans	1				
Forsythia x intermedia	*Cladosporium cladosporioides*	FZ32	I	I	I	I
"FZ" and "F"	*Phoma macrostoma*	F29	I	I	I	I
	Botryosphaeria sarmentorum	F27	I	I	I	I
	Plenodomus influorescens	F23	N	I/	I/	I/
	Glomerella cingulata	FZ33	N	I/	I/	I/
	Epicoccum nigrum	FZ18	I	I	I	I
	Colletotrichum rhombiforme	FZ12	N	N	I	I
	Fusarium lateritium	F17	N	N	N	I/
	Botryotinia fuckeliana	FZ22	N	G	G	G
	Glomerella acutata	F60	N	N	I	I
	Phomopsis sp	F34	N	N	N	G
	Phoma viburnicola	F39	N	N	I	I
	Cytospora sp.	F42	N	I	I	I
	Botryosphaeria sarmentorum	F27x	N	N	N	N
Site : Privet (Cruick)	*Phoma exigua*	PC13	N	I	I	I
Ligustrumvulgareaureovariegatum	*Epicoccum nigrum*	PC7	N	N	I	G
"PC"	*Phoma macrostoma*	PC21	I	I	B	B
	Lewia infectoria	PC8	I	I	I	G
	Mycosphaerella coacervata	PC28	N	N	I	I
	Phoma viburnicola	PC5	I	I	I	I
	Diaporthe viticola	PC18	I	B	B	G
Site : Privet (Side)	*Phomopsis sp.*	PS20	N	I	I	I
Syringaemodi	*Cladosporium cladosporioides*	PS24	N	N	I	I
"PS"	*Sordariomycetes sp.*	PS31	N	N	N	N
	Cadophora luteo-olivacea	PS30	N	N	I	I

	Diaporthe viticola	PS12	N	I	I	I
	Phoma macrostoma	PS10	N	N	I	I