IMPACT AND EPIDEMIOLOGY OF A FUNGAL DISEASE OF ORNAMENTAL HORNBEAM (Carpinus betulus L.) TREES IN NORTHERN ITALY *

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* Research supported by a grant of the Comune di Torino - Divisione Verde Pubblico.

Keywords: Cytospora, Endothiella, decline, cankers, infection.
Parole chiave: Cytospora, Endothiella, deperimento, cancri, infezione.

Abstract
In 2002 a new disease of hornbeam (*Carpinus betulus* L.) trees was noticed in the City of Turin (Italy). On 90% of symptomatic hornbeams, we found pycnidia belonging to two different fungal taxa, identified as *Cytospora* sp. and *Endothiella* sp. Pycnidia were observed on the bark, in association with necrotic areas.
The main goal of this study was to investigate the impact and the epidemiology of this new disease of hornbeam trees. We (i) assessed the incidence of the disease and its evolution through periodic phytosanitary surveys in the City of Turin; (ii) determined the pathogenicity of *Cytospora* sp. and *Endothiella* sp. through inoculation experiments; (iii) characterized populations of both fungal taxa to infer possible ways of infection.
In the City of Turin, the incidence of mortality was 12% in 2004, and over 20% in 2007. Incidence of symptomatic trees increases by 12% in three years.
Inoculation tests on *C. betulus* trees using mycelium and conidial suspensions, confirmed pathogenicity of the two fungal species. However, conidial suspensions were rarely infective.
Pairing tests on 36 and 35 isolates of *Cytospora* sp. and *Endothiella* sp. proved high genetic variability in *Cytospora* sp. populations. PCR-RFLP analysis performed on a sub-sample of isolates not only confirmed results of pairing tests for *Cytospora* sp. but also showed significant genetic variability in populations of *Endothiella* sp. These results, combined with the above results of inoculations, suggest infections of these two pathogenic fungi may not rely on conidia, but rather they may occur through some other, undetected, ways.

1 Introduction

The European hornbeam (*Carpinus betulus* L) is widely distributed in Central Europe and in Italy, where is often present in the relict plain forests in association with the bay-oak (Carpinion alliance) (Pignatti, 1998).
The hornbeam is a rustic and easy-fitting species largely used in urban environment as ornamental. Ecological characteristics of this tree species include tolerance to air pollution and drought, and prompt reaction to the pruning.
In 2002 an unknown form of dieback was noticed on hornbeam in the City of Turin (Italy).
The symptomatic trees showed thin crown, localized mortality of shoots and branches, cankers and lesions on the bark. Tree mortality generally occurs in a short period of time.

Two types of fungal fructifications were observed on 90% of symptomatic hornbeams in association with necrotic areas on the bark. Bright red-orange, roundish-ovoidal pycnidia (about 2-10 mm-diameter) containing hyaline, lunate conidia as well as yellow-orange, spherical pycnidia (about 2 mm-diameter) containing cylindrical conidia were present singly or simultaneously on a same tree or even on the same branch. No sexual fructifications were noticed.
Based on micromorphology of pycnidia and on nucleotides sequences of ITS regions, fungi isolated were deemed to belong to the genera *Cytospora* and *Endothiella*, respectively.
The ITS sequence of morphotype identified as *Cytospora* sp. showed similarity values of the 90- 91% with ITS sequence of *Eutypella cerviculata* and of the 86-87% with *Eutypa lata* and *Libertella blepharis.* Morphotype identified as *Endothiella* sp. showed similarity of 94-95% with *Cryphonectria radicalis* and of the 94% with *C. parasitica* and *Endothiella girosa.*
In 2006, a similar syndrome was reported in Lombardy (Saracchi *et al*. 2007).
The main goal of this study was to investigate the impact and the epidemiology of this new disease of hornbeam trees. We (i) assessed the incidence of the disease and its evolution through periodic phytosanitary surveys in the City of Turin; (ii) determined the pathogenicity of *Cytospora* sp. and *Endothiella* sp. through inoculation tests; (iii) characterized populations of both fungal taxa to infer possible ways of infection.

2 Materials and Methods

2.1 Phytosanitary surveys

On the basis of preliminary surveys in the city of Turin, 300 trees growing on 12 different sites with disease incidence exceeding 30% were analyzed in autumn of 2004 and in summer 2007.
Health condition for each tree was scored as follows: healthy tree/ declining tree/ dead tree.
Disease incidence was calculated as number of declining and dead trees on the total number of hornbeams examined. Frequency of trees infected by *Cytospora* sp., *Endothiella* sp. and by both species was also calculated.
Data collected in 2004 and in 2007 were compared to determine the evolution of disease incidence.
During the surveys of 2007, we also examined health conditions of young hornbeams planted to replace dead trees.
Bark samples with pycnidia were collected for fungal isolation.

2.2 Pathogenicity tests

Pathogenicity experiments were performed on *Carpinus betulus* by inoculating (i) *Cytospora* sp., (ii) *Endothiella* sp. or (iii) both fungi.
In 2003, 50 two-year old seedlings were inoculated using conidial suspensions sprayed (i) on artificial wounds created on the stem and (ii) on lesions caused after the petiole removal.
In 2007, pathogenicity tests were performed on 50 six-year old hornbeams using mycelial plugs inoculated on artificial wounds on the stem, 10 cm above the root collar. In both experiments 15 plants were wounded and non-inoculated. These plants were used as controls.

During the two vegetative periods following the inoculation, hornbeams were weekly controlled for the presence of cankers and/or pycnidia.
The infection frequencies of *Cytospora* sp. and *Endothiella* sp. as well as the average length of the necrotic areas caused by the two fungi were calculated. A Yates corrected chi-square test in contingency table was performed to test differences between infection frequency of the two fungi ($p<0.05$). Mann Whitney U test ($p<0.05$) was used to assess differences between the two fungi in terms of length of the associated necrotic areas. The same statistical analysis was performed to test differences between the two fungi in terms of increment of lesion size over 130 days.

2.3 Population studies

Pairing tests were performed among 36 isolates of *Cytospora* sp. and among 35 isolates of *Endothiella* sp. collected from 7 and 5 different sites, respectively. The isolates from a same site were paired in all possible combinations on MEA plates and incubated at room temperature for about one month. Pairings of mycelium from a same isolate were used as controls.
Vegetative compatibility or incompatibility between isolates was determined for each pairing through observation of the total fusion of hyphae or the formation of a *barrage* zone between mycelia, respectively.
Percentage of isolates belonging to different vegetative compatibility groups on the total number of isolates examined was calculated for each site.
Based on results of pairing test, a subset including six isolates for each fungal species was selected for PCR-RFLP (Restriction Fragment Length Polymorphism).
DNA was extracted from mycelium through the CTAB-based method described by Gardes and Bruns (1993).
The Internal Transcribed Spacers region (ITS) was amplified using primers ITS1F (Gardes & Bruns, 1993) and ITS4 (White *et al.,* 1990). The PCR amplification parameters were: 1,5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 53°C, 3 min at 72°C and a final step at 72°C for 10 min.
The amplification of the Intergenic Spacer region (IGS) was obtained using LR12R and invSR1R primers (Vilgalys & Gonzales, 1990, Vilgalys *et al.,* 1994). The PCR amplification program was as follows: 3 min at 94°C, 15 cycles of 10 sec at 94°C, 3 min at 65°C, 15 cycles of 10 sec at 94°C, 3,20 min with a 20 sec-increment each cycle.
Electrophoresis in 1% Agarose gel in 1x Tris-Borate EDTA Buffer (TBE) at 3.4 $V \cdot cm^{-1}$ for 1h 20 min was performed to analyze PCR products.
RFLP analysis was conducted on the ITS amplicons with *HinfI* and on IGS amplicons with *HinfI*, *AluI* and *Hin6I*. A 2% Agarose gel electrophoresis in 1x TBE was carried out at 4 $V \cdot cm^{-1}$ for 3 h 30 min to analyze RFLP patterns.

3 Results and Discussion

The disease incidence was 42% in the 2004 and 54% in the 2007. Tree mortality was 12% in 2004 and over 20% in 2007. Symptoms of tree diseases were observed in 2% of the young hornbeams planted to replace dead trees. Majority of hornbeams that were declining in 2004 were scored as dead in 2007. Data suggest the disease is currently in severe evolution (Tab. 1).

Tab. 1 – Health conditions of hornbeam trees in the city of Turin during surveys of 2004 and 2007. Number refers to percentage on the total number of trees examined.

	2004 survey	2007 survey	Evolution
Healthy trees	58 %	46 %	- 12 %
Declining trees	30 %	31 %	+ 1 %
Dead trees	12 %	23 %	+ 11 %
Disease incidence	**42 %**	**54 %**	**+ 12 %**
Trees infected by *Cytospora* sp.*	40 %	57 %	+ 17 %
Trees infected by *Endothiella* sp.*	50 %	30 %	- 20 %
Trees infected by both fungi*	10 %	13 %	+ 3 %

* Percentage refers to declining and dead trees.

While neither disease symptoms nor signs were observed on trees inoculated in 2004 with conidial suspensions, fructifications and necrotic lesions were detected, with a frequency of 100% for *Cytospora* sp. and of 67% for *Endothiella* sp., on trees inoculated in 2007 with mycelial plugs. Infection frequency of trees inoculated with the two fungi differed significantly (χ^2= 4.69, d.f.=1, p=0.03).

At the end of vegetative season of 2007 the average length of lesions caused by *Cytospora* sp. and by *Endothiella* sp. was about 16 cm and 3 cm, respectively (Fig. 1). On the inoculated trees, lesions caused by *Cytospora* sp. reached 56 cm of length. Lesions length on trees inoculated simultaneously by both fungi was similar to that caused by each fungus in single inoculations. Size of lesions caused by *Cytospora* sp. was always higher than that caused by *Endothiella* sp. (p<0.05).

No symptoms of disease were detected on the hornbeams used as controls (not inoculated).

No foliage symptoms have been noticed so far in inoculated trees.

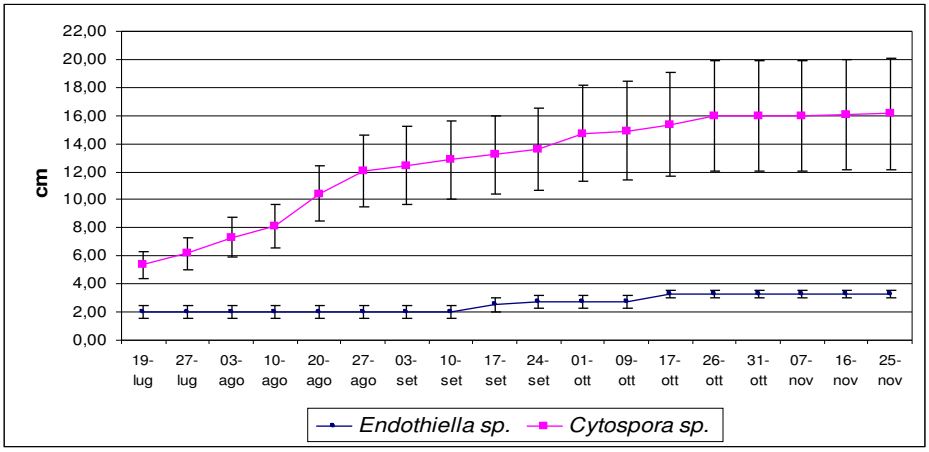
Although the infective role of conidia was not demonstrated, these preliminary results confirmed the pathogenicity of both fungal species. Based on data obtained from phytosanitary surveys and inoculation tests, *Cytospora* sp. proved to have greater colonization ability and aggressiveness than *Endothiella* sp. Data also indicate little interaction and synergistic activity between the two fungi.

Pairing tests and molecular analysis proved high genetic variability in population of both fungal species.

After pairing tests, the percentage of *Cytospora* sp. isolates belonging to different vegetative compatibility groups ranged from 67% to 100% depending on site. Incompatibility reactions were extremely clear for *Cytospora* sp., they were not for *Endothiella* sp.

As concerns *Cytospora* sp., RFLP patterns confirmed most isolates from a same site were different genotypes. The same was true for *Endothiella* sp.

Fig. 1 – Average length of lesions caused by *Cytospora* sp. and *Endothiella* sp.
At each period, size of lesions associated with the two fungi differed significantly (Mann Whitney U test, p<0.05).



4 Conclusions

Cytospora sp. and *Endothiella* sp., whose ability to cause cankers and necrosis on hornbeam trees is here demonstrated, proved to be wound pathogens associated to hornbeam decline. Furthermore, their ability to colonize young and healthy trees as assessed both in the field surveys and in the pathogenicity tests, suggest they may not behave exclusively as weakness pathogens. Nevertheless, the absence of significant reports of the disease in the forest may indicate the stresses typical of urban environment are triggering factors in the epidemiology of this disease.

Conidia were never infective. This result is consistent with results of population studies, according to which most infections in the field are caused by different fungal genotypes.

We propose infections not occur through conidia but in some other undetected ways.

Aknowledgements

We acknowledge Sara Piani for technical assistance.

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