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Disease gradient of the anthracnose agent *Apiognomonia quercina* in a natural oak stand

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Summary. Patterns of spore dispersal of the fungal pathogen *Apiognomonia quercina* and its anamorph *Discula quercina* were investigated over two consecutive growing seasons in a natural mixed stand of *Quercus cerris* and *Q. pubescens* trees located in a inland area of Tuscany, at an altitude of 400 m a.s.l. To measure spore dispersal, a transect was laid out in the stand to serve as an inoculum source. The rate of inoculum dispersal (conidia and ascospores) was quantified by means of spore traps positioned at 10, 100, 500 and 1000 m from the southern end of the transect. The disease gradient was also assessed by determining the disease incidence on selected trees at the same distances from the transect. The amount of inoculum detected decreased steeply with the distance from the transect. Disease incidence was inversely correlated with the disease gradient, i.e. with the distance from the inoculum source, and it was much higher at the shorter distances. The level of spore dispersal was related to both the distance from the infection foci and the sporulation time. The experimental approach constituted a valid means for describing and understanding the dynamics of windborne diseases in forests.

Key words: inoculum dispersal, disease incidence, oak wood, *Discula quercina*, *Quercus* spp.

Introduction

The ascomycete *Apiognomonia quercina* (Kleb.) Höhn (anamorph *Discula quercina* [West.] Arx) is the causal agent of oak anthracnose. Like several related members in the *Apiognomonia* (Gnomoniaceae, Diaporthales) complex, that cause similar anthracnose diseases to trees and shrubs worldwide, this pathogen induces leaf spot, shoot blight and cankers (of varying length on current-year and 2-5-year-old twigs) to some species of the genus *Quercus* in their native and planted ranges. The first noticeable symptom of oak anthracnose is leaf blotching, the formation of small, brown spots at the start of the growing season. Later infections cause discrete reddish-brown spots, variable in size, with an irregular margin, located in the angle between the midvein and the lateral veins (angular spots). As the spots grow larger they coalesce to form large necrotic areas, causing leaf withering and distortion. The fungus also spends a part of its life as a latent endophyte in the inner tissue of a number of healthy and declining oaks species (Ragazzi *et al*., 1999a).

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In Italy oak anthracnose was reported with high incidence in the 1990s in both mixed stands (*Quercus* / *Fagus; Quercus* / *Castanea; Quercus* / *Ostrya*) and pure stands, and on isolated trees. The most susceptible *Quercus* species were: *Q. cerris* L., *Q. frainetto* Ten., *Q. pubescens* Willd., *Q. robur* L., *Q. suber* L., and to a less degree *Q. macrolepis* Kotsc. and *Q. troyana* Webb. (Ragazzi *et al*., 1999a).

The impact of the disease has been considerable in areas with a semi-arid/temperate climate. In the Italian Peninsula *A. quercina* is found from the southernmost regions, e.g. Sicily (37–38° N, 13–15° E) to regions with a temperate Mediterranean climate like Tuscany (43–44° N, 10–12° E); but it has not been reported from the more northerly regions, beyond the Apennines. In those areas where it occurs, *A. quercina* is isolated either as an endophyte (from healthy tissue) or as a pathogen (from necrotized tissue) with a very high frequency (from 97% of all locations). Its whole range is characterised by seasons with widely differing levels of precipitation. The experimental evidence indicates that the amount of rainfall is decisive in determining the biology and the phenology of the host trees, and hence the aggressiveness of the pathogen (Moricca and Ragazzi, 2008).

Early infections occur in spring, when temperatures rise above 18–20°C, which is sufficient for the perithecia and acervula to mature and for the ascospores and conidia to be released. Optimal temperatures for ascospore and conidia germination, as well as for host infection and colonization, are between 22 and 24°C; percent germination decreases drastically at temperatures below 16°C (Ragazzi *et al*., 1999d). The endophyte survives the winter in infected leaves and branches that break off and fall on to the litter or that remain attached to the tree. Primary infections of the buds, shoots, and expanding leaves are caused by overwintering sexual spores discharged by hypophyllous perithecia immersed in these plant organs. The conidia ooze out of the acervula in white masses repeatedly during the growing season, when temperature and rainfall conditions are favourable, and they give rise to cyclic infections that intensify the disease and further increase the amount of inoculum in the environment. The asexual propagules of the pathogen are thus the most important stage for the dissemination of the pathogen and for tree infection. According to von Arx (1970), the sexual and the asexual forms of the fungus are sympatric in that they inhabit the same host, but they are also partly allotopic since each form settles in anatomically different tree organs. Both ascospores and conidia infect leaves, buds, shoots and twigs, but the teleomorph preferentially colonizes the leaves, buds and shoots, while the anamorph is found not only on the leaves and buds but also on the twigs (bark and wood) (Ragazzi *et al*., 1999a). The ascospores and conidia are dispersed to new infection sites by means of wind, water (splashing rain) and infected seeds. Insects are known vectors of anthracnose fungi (Fokunang *et al*., 2000) and are also thought to have a role in the dispersal of the oak anthracnose agents at Italian latitudes (Tiberi *et al*., 2002).

The oak anthracnose-tree interaction has been extensively investigated in the past few years for a number of epidemiological aspects. The biology of the fungus (infection ability, inoculum density, conidia production, pathogenic variation, host preference, isolation frequency in relation to particular tree organs, tree phenology and physiology) has been the subject of thorough-going investigations (Ragazzi *et al*., 1999a, b, c, d; 2000; 2001; 2002; 2003; Anselmi *et al*., 2004; Ragazzi, 2005).

The present work was intended to continue and supplement these studies. Its aim was to determine the disease gradient of *A. quercina* in a stand of *Q. cerris* mixed with sporadic *Q. pubescens* individuals when the trees were exposed to naturally disseminated ascospores and conidia. The stand in which the experiment was carried out consisted of both healthy and diseased individuals of the two oak species mentioned.

Materials and methods

The study was carried out at Ulignano (UTM coordinates x: 1655324,73; y: 4813251,52) in the province of Pisa (Tuscany), in a mixed stand consisting mainly of *Q. cerris* with a small proportion of *Q. pubescens*. The stand was located at an altitude of about 400 m a.s.l., and included both healthy and diseased trees, with an average height of 15 m.

A transect measuring 250 by 50 m and containing 68 *Q. cerris* and 14 *Q. pubescens* trees was traced out in the stand along the main axis (north-south), which also corresponded to the prevailing wind direction (as measured by a weather-vane raised 3 m from the ground). The transect was oriented so as to minimise the likelihood that trees with spo-

rulating lesions growing outside the transect would disperse any propagules to either the spore traps or to the branches of the oaks used to calculate the disease gradient. Such a possibility was in any case unlikely as the oak population extended almost entirely along a north-south line within the transect borders, with just a few individuals that remained outside its eastern and western sides, interspersed within the rest of the Mediterranean maquis vegetation. To definitely exclude any contribution of propagules from trees located outside the transect, a preliminary test was undertaken in the growing season immediately preceding the two-year trial. In collaboration with the forest service of the local municipality that manages the stand, spore traps were set up exactly as in the present experiment (see below); in addition, four spore traps were positioned externally to the transect, approximately 50 m to the east (2 spore traps) and to the west (2 spore traps) of the virtual line extending the eastern and western sides of the transect from its southern end, at distances of 10 m and 500 m. This trial showed that the amount of propagules contributed from sources outside the transect was negligible.

Trees suffering from decline were deemed to be the sources of these ascospores and conidia since fungal endophytes begin sporulating on woody plants when these are under stress or senescent (Wilson and Carroll, 1994; Faeth and Hammon, 1997).

Two spore traps each (Model 60A, Ted Brown Associates CA, USA) were set up 10, 100, 500 and 1000 m from the southern end of the transect to trap the ascospores and conidia and to determine the mass of inoculum. The spore traps were operated from 12 noon to 2 p.m. once every ten days (from day 10 to day 40, giving four readings).

The mass of inoculum (ascospores plus conidia) was determined along a virtual line perpendicular to the wind direction 10, 100, 500 and 1000 m from the end of the transect, along which 10 declining *Q. cerris* and 5 declining *Q. pubescens* trees were inspected. These trees exhibited numerous cankers. The cankers were counted on 8 branches per tree, 2 branches at each point of the compass. The fungus was isolated from the cankers, according to the data shown in Table 1.

The transect was laid out in April 2004, and the tests were carried out in 2004 and in 2005. Spore counts were started in April 20, 10 days after the transect was laid out, and ended in May 20. Relative humidity and temperature data were also recorded and are shown as average values for the two years of the trial (Fig.1). Light intensity was measured under canopy and in clearings at 1 meter from the soil by means of a Vianello Photovolt Model 200L. (Italy)

Inoculum concentration was calculated according to the following formula:

Ascospores - conidia / volume = total number of propagules / (rpm) × (k) × (min)
where K is a constant which for this type of spore trap is equal to 0.050.

Table 1. Number of cankers on *Quercus cerris* and *Quercus pubescens* twigs from which *Apiognomonia quercina* was isolated.

Tree No.	Twig diameter			
	Quercus cerris		*Quercus pubescens*	
	<2 cm	>2 cm	<2 cm	>2 cm
1	15	18	14	16
2	12	15	10	12
3	11	13	9	11
4	12	16	13	13
5	12	16	14	15
6	17	20		
7	11	13		
8	8	11		
9	10	12		
10	10	14		

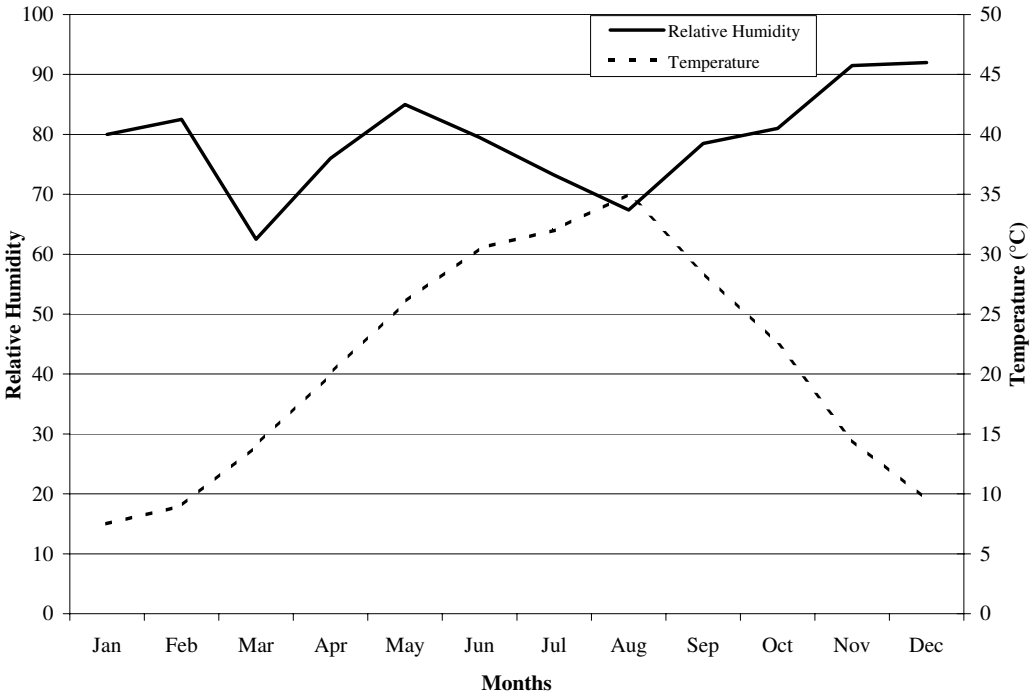

Fig. 1. Average monthly temperature and relative humidity recorded at Ulignano (Pisa) in 2004 and 2005.

The disease gradient was calculated on the basis of the number of sporulating cankers. All cankers on twigs were taken to be sporulating cankers, following the exponential model of Kiyosawa and Shiyomi (1972), since acervula of the anamorph were always observed on the twigs. In this connection, the rate of inoculum increase was in accordance with the Kiyosawa and Shiyomi (1972) exponential model. This model predicts a rapid increase in the disease, even if this seems scarcely compatible with a real biological system. Only in the very early stages does a biological system develop according to an exponential model, and it is just in this connection that this short-term experiment has to be viewed.

Results and discussion

Of the 68 *Q. cerris* trees in the transect, 44 showed signs of decline, and from 32 of these *A. quercina* was isolated from the buds and leaves, as well as from one and two-year-old twigs. Of the 14 *Q. pubescens* trees, 8 were in decline, and from 5 of these *A. quercina* was isolated as an endophyte, the same tree-organs being infected as on *Q. cerris*.

Apiognomonia quercina was isolated on PDA after culturing at about 20–22°C in the dark. Mycelial primordia were visible to the naked eye at 4 days of incubation and gave rise to pale brown colonies. Mycelium consisted of hyaline, septate and branched hyphae compacted to the medium. Acervula were about 140–160 μm in diameter and differentiated at 5 days of incubation on impoverished PDA (6 g l^{-1} potato dextrose broth [PDB] + 20 g l^{-1} agar). Conidiophores measured 13–35×10–21 μm and were hyaline, septate, single, sometimes branched at the base, straight or slightly curved, and elongated at the tip. Conidia were 9–14×3.5–6 μm, hyaline, oblong, ellipsoid, one-celled, with a smooth, thin wall, a blunt tip and a somewhat truncated base.

Apiognomonia quercina differentiates the perithecia on abscissed leaves, while the anamorph *D. quercina* produces the acervuli on the bark of infected twigs, and occasionally on the leaves. The number of infective propagules (conidia and ascospores) diminished with the distance from the transect, as reported in the literature for various pathogens (Gregory, 1968; van Arsdel, 1980; Roche

et al., 1995; Xu and Ridout, 1998). Ten days after the start of sporulation, the average number of propagules trapped in the spore traps decreased from 49,300 propagules/m^3 air at 10 m from the transect to 16,200 propagules/m^3 at 1000 m. After 40 days, the corresponding figures were 16,200 propagules/m^3 at 10 m and 2400 propagules/m^3 at 1000 m. For all the four sampling times the greatest number of propagules was always recorded at 10 m from the transect: 49,300; 37,200; 19,600; 16,200 per m^3 of air respectively (Fig. 2).

The difference between sampling areas and sampling times was always highly significant, at P=≤0.01 and 0.05 (Table 2).

The disease gradient and the corresponding non-linear regression are shown in Fig. 3. As the distance from the inoculum source increased, the number of cankers per tree decreased. The decrease can be expressed by the equation $Y=ae^{-bx}$, where Y is the number of cankers per tree; a= a constant (the Y intercept); e= the base of the natural logarithm; b= a rate constant; and x= the distance from the inoculum source. The coefficient of regression always remained close to one.

An increase in the distance of the inoculum source from 10 to 1000 m decreased the number of cankers by 71.2%.

The count of propagules dispersed from the *Q. cerris* and *Q. pubescens* trees in the transect decreased with increasing distance from the transect, and also from the first to the last sampling time (10, 20, 30 and 40 days).

With the increase in distance, the disease gradient decreased; this decrease was more marked between 100 and 500 m than between 500 and 1000 m.

The disease gradient values were relatively flat. This was presumably related to the lower rate constant value b, which was due to the fact that the propagules were not dispersed in a linear trajectory but their path was affected by wind currents and by the topography (hilly nature) of the terrain. It should be borne in mind however that the disease gradient generally depends not only

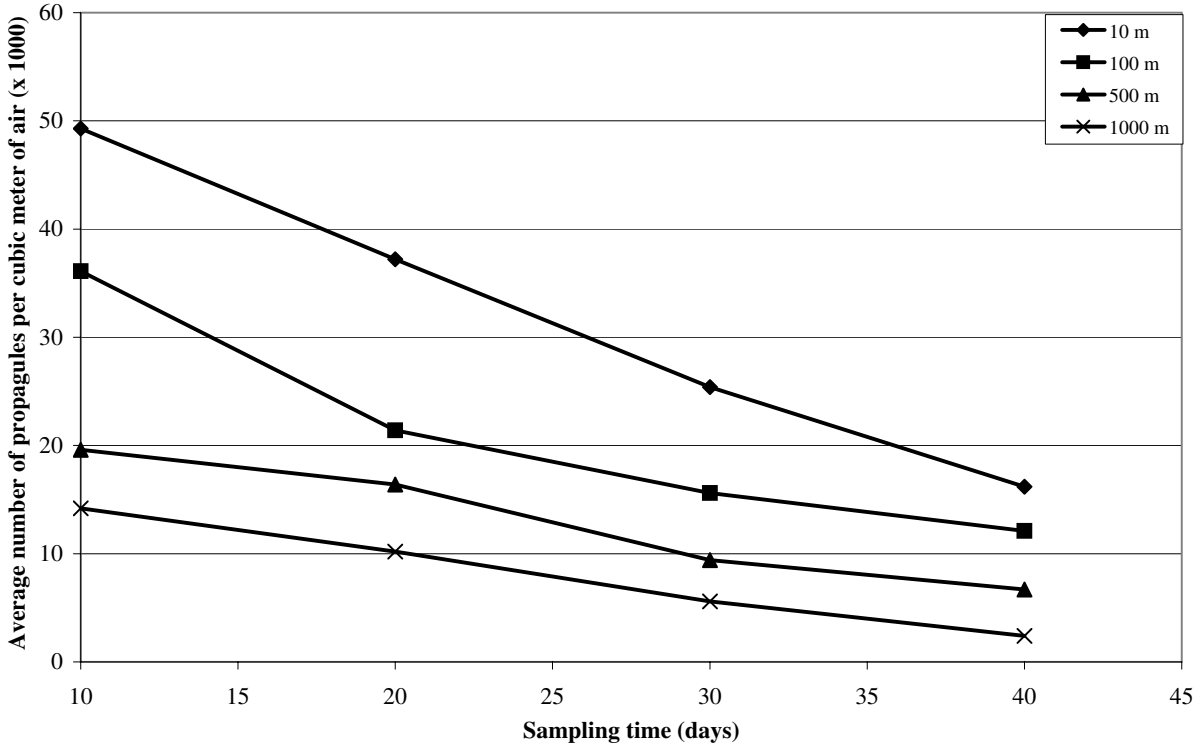

Fig. 2. Average number of *Apiognomonia quercina* propagules (ascospores and conidia) recorded in 2004 and 2005 per m^3 of air (×1000) in relation to distance from the inoculum source.

Table 2. ANOVA of the number of *Apiognomonia quercina* propagules released from *Quercus cerris* and *Quercus pubescens* trees.

Variation	Df	Deviance	Variance	P^a
Total	8	2113.59		
Between sampling areas	3	1809.11	603.03	498.37**
Between sampling times	3	302.06	100.68	83.20**
Error	2	2.42	1.21	

[a] Significant at P=≤0.01 and 0.05.

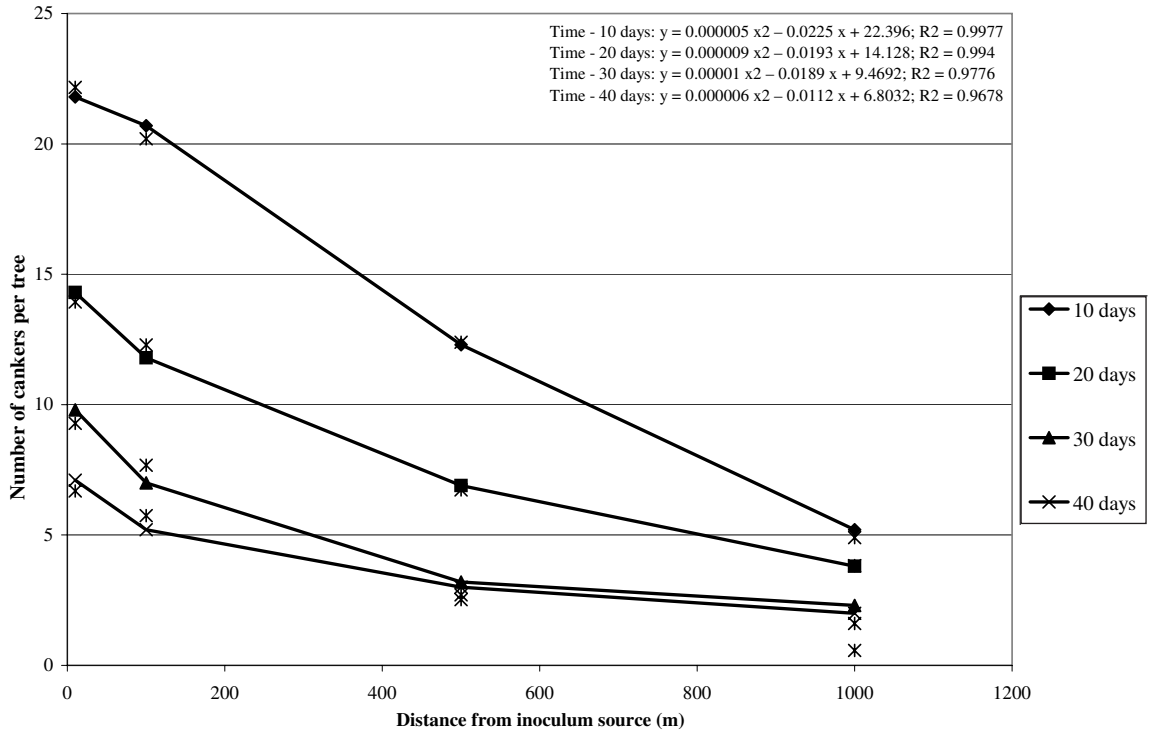

Fig. 3. Disease gradient of *Apiognomonia quercina* on *Quercus cerris* and *Quercus pubescens*. Average number of cankers per tree was determined on the twigs of 15 trees (2 twigs per compass point = 8 twigs per tree) at each of four sampling points (10, 100, 500 and 1000 m from the inoculum source).

on the factors mentioned above, but also on the biology of the infective agent (germination, mode of penetration, fruit body formation, spore dispersal) as well as on the spatial aggregation of infected trees (infection foci).

The ascospore dispersal of the black knot agent of cherry (*Prunus cerasus* L.) *Apiognomonia morbosa* (Schwein.:Fr.) Arx, taking into account rainfall (mm), temperature (°C) and duration of wet conditions (h), was expressed by the following multiple regression (Mc Fadden-Smith, 2000):
log (ascospores cm^{-2} +1)=-4.775+0.325 (rain)+0.763 (temperature)–0.0047 (rain^2)–0.023 (temperature^2)+0.032 (wetness) (r^2=0.77).

The release of *Discula* sp. spores from infected *Fagus crenata* Blume individuals in Japan reached a peak in May (Sahashi *et al*., 2000). The disease gradient of *Cronartium quercuum* f. sp. *fusiforme*

Burds. & G.A. Snow decreased by 90% as the distance from the infection sources increased from 3.7 to 152.4 m (Schmidt *et al*., 1982).

The incidence and severity of oak anthracnose depends on the weather conditions (wind, rainfall, temperature and humidity) during the growing season (Anselmi *et al*., 2004). Under experimental conditions, the ascospores and conidia of *A. quercina* show a high infection rate at two day/night regimes: 16/8 h, 20,000/0 lux, 22/18°C and 40/65% RH; and 14/10 h, 10,000/0 lux, 18/15°C and 50/75% RH. These are conditions that at Italian latitudes frequently occur in spring (Ragazzi *et al*., 1999c; 1999d) and that are optimal for *A. quercina* ascospore and conidia production. These conditions were also in line with the temperature, relative humidity and light intensity data recorded at Ulignano during the study in March and April, the months preceding sporulation (Table 3).

The amount of 100,000 conidia ml^{-1} is optimal for leaf infection, producing the highest percent leaf necrosis in test oaks. Any decrease or increase in the spore concentration from this amount lowers the percent infected leaf area (Ragazzi *et al*., 1999a).

Spore trapping revealed that in a controlled environment conidia production peaked at 23,000 conidia/m^3 air with a day/night cycle of 12/12 h, 25,000/0 lux, 25/15°C, and 50/75% RH for 14 days from the start of conidiogenesis, with a daily peak being recorded between night and early dawn (21.00 h and 06.00 h) (Ragazzi *et al*., 1999a). These findings have considerable epidemiological importance, as the pathogen biomass increases especially as a result of secondary mid-season infections caused by wind-borne conidia. The asexual form develops in and around the canker lesions produced by the primary inoculum (ascospores) on the shoots and twigs (though acorns are also infected) and causes the sudden increase in disease severity as the temperatures rise.

There is experimental evidence that the distribution, abundance and colonization of fungal endophytes decrease along an altitudinal gradient, probably as a result of both microclimate changes and interactions with other fungi (Toti *et al*., 1993; van Maanen *et al*., 2000). A study on dogwood anthracnose caused by *Discula destructiva* Redlin in the United States (Chellemi, 1992) found that altitude also had a slight effect on the incidence of this disease.

Conclusions

The anthracnose fungi include a number of serious emerging and invasive tree pathogens that cause defoliation and branch dieback, and disfigure the tree if the attack is particularly severe. In Italy the oak anthracnose holomorph causes blight on the twigs, buds, shoots and leaves. Previous investigations on the ecology and the life cycle of this pathogen suggested that it had a high potential for epidemic spread (Ragazzi *et al.*, 1999c). The present research therefore explored the rate of dispersal of this windborne agent in relation to its distance from the host, and the sporulation time.

In conclusion it can be stated that the number of airborne propagules was related to 1. the distance of the inoculum source from the host, and 2. the trapping inspection time (at the 10-day time interval). Windborne spores of endophytes are important in the epidemiology of diseases, as shown by Sahashi *et al*. (2000) for *Discula* sp. on *Fagus crenata*; by Johnson and Whitney (1992) on black spruce, and by Toti *et al*. (1993), on European beech.

Pathogen biomass (here expressed as the number of propagules per m^3 of air) is an important factor that may determine whether trees become infected,

Table 3. Average values of climatic parameters (temperature, relative humidity and light intensity) recorded at Ulignano (Pisa) in March and April of 2004 and 2005.

Parameter	Months	
	March	April
Temperature (°C)	14	20
Relative humidity (%)	62.5	76
Light intensity (lux)	11,000 (under canopy)	12,000 (under canopy)
	20,000 (in clearings)	22,000 (in clearings)

even at a distance of 1000 m. From an epidemiological point of view, however, since every infected oak represents a potential source of inoculum for a non-infected tree, all inoculum sources, at whatever distance from their potential hosts, be they *Q. cerris* and *Q. pubescens*, as here, or any other susceptible oak species, must be carefully inspected. Since the risk of infection was high even when the potential inoculum source was 1000 m away, it is advisable to establish new stands at a greater distance from such sources. The current surface area of oak forests in Italy is 884,600 ha, which is expected to increase in the near future, since these species are cultivated for multiple purposes: hydrogeological protection, landscaping (amenity trees), and especially timber production. The wood of many oaks has significant commercial value, being increasingly used for firewood, pulp, particle boards, bio-energy production, etc., and this has recently increased the demand for such wood, leading to a renewed interest in oak cultivation (Ciancio *et al*., 2002). When choosing a management system (high forest, coppice) it will be important to consider the proximity of the stand to potential sources of *A. quercina*.

Despite the foregoing, however, the findings of the study have validity only on a local or regional scale and cannot automatically be generalized. As environmental conditions obviously vary substantially in different areas, they directly impact the epidemiology of diseases. The life cycle components of both the tree (bud burst, foliation, shoot sprouting, green tissue hardening) and the pathogen (latent period, infectious period, lesion growth rate, multiplication rate), upon which disease dynamics largely depend, are under the constraints of local or regional weather conditions. Members of the *Apiognomonia* complex are however reported to be well suited to a wide range of temperatures (from 3 to 30°C) (Neely and Himelick, 1967; Wilson and Carroll, 1994; Sinclair and Lyon, 2005), and it is therefore expected that the findings of the present study will also be valid in other Mediterranean countries with a similar climate.

Epidemiological studies like the present are important to improve disease forecasting systems on a regional and local scale, as well as, where possible, to suggest control measures. A first such measure in the case of *A. quercina* could be to collect and burn all fallen leaves and twigs on which the fungus differentiates the teleomorph. Genotypes of sexual origin (ascospores) increase pathogen fitness and accelerate the rate at which epidemics break out and spread (Zwankhuizen *et al*., 1998). Elimination of infected plant material can therefore be expected to be important in preventing epidemic outbreaks. Felling severely infected trees would have multiple benefits: it would reduce the number and density of infection points (infection foci), diminish the pathogen biomass, and increase the level of genetic resistance in the plant population by removing the most susceptible genotypes. Where economically feasible (as with high-value trees or in recreational spaces), the removal of branches in oak trees could increase air movement through the crown, causing a more rapid drying of the succulent shoots and leaves, which would hamper pathogen sporulation.

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