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EPIDEMIOLOGY AND CONTROL STRATEGIES APPLIED TO ASH DIEBACK AND CHESTNUT INK DISEASE

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Ai miei genitori

Enthusiasm is one of the most powerful engines of success. When you do a thing, do it with all your might. Put your whole soul into it. Stamp it with your own personality. Be active, be energetic, be enthusiastic and faithful, and you will accomplish your object. Nothing great was ever achieved without enthusiasm.

Ralph Waldo Emerson

Summary

Main goal of forest diseases' management is to reduce economic, biological and aesthetic damages and biodiversity loss caused by plant parasites. The many strategies used can be grouped under two main actions, prevention (prophylaxis in some early writings) and therapy (treatment or cure). Prevention is limited primarily by the lack of knowledge of the organisms involved, including host plants. Mathematical models have been used to extend the understanding of plant disease epidemiology on a number of fronts, providing an opportunity for a more rational use of resources on expensive field trials and representing a step towards more sustainable control measures. From a curative point of view, current efforts by scientists have focused on developing diseases management (Pest Management = PM) concepts in order to balance the benefits of pesticides with the ecological concerns of their residues contaminating the environment.

In this thesis, the two PM principles were applied from an innovative point of view on two case studies: ash dieback caused by *Hymenoscyphus fraxineus*, which can be considered the most serious disease for *Fraxinus* genus in Europe, and chestnut ink disease, caused by *Phytophthora cambivora* and *P. cinnamomi*.

In the first part of the thesis, the two diseases are introduced, in order to permit the evaluation of similarities and differences (chapter I).

Subsequently, from chapter II to chapter V, the experimental trials performed are described. In particular, in chapter II a study of the ecological niche of *H. fraxineus*, with the characterization of the environmental variables associated with naturally infected zones, is reported. This procedure was realized with Species Distribution Models (SDM), widely utilized in the ecological field and only recently applied to plant pathology. The presence of the pathogen was highly correlated to

three summer predictors: abundant precipitation, high soil moisture and low air temperature, in comparison with the averages of the study area. The ensemble forecasting technique was then applied to obtain a prediction of the potential distribution of the pathogen at European scale, considering the distribution maps of *Fraxinus excelsior* and *Fraxinus angustifolia,* susceptible to the parasite. At last, an innovative method of network analysis permitted to identify the suitable areas that are not reachable by the pathogen with a natural spread.

Chapter III reports a study conducted to evaluate six fungicides for their potential to control ash dieback. Initially, *in vitro* tests of the active ingredients against five different strains of the pathogen indicated thiabendazole, propiconazole and allicin as the most effective fungicides, with lower median lethal doses than procloraz. In contrast, copper sulphate and potassium phosphite were totally ineffective. Subsequently, the antifungal activities of the best three compounds were investigated *in planta* against *H. fraxineus* by trunk injection on European ashes inoculated with an indigenous strain. The test was preceded by preliminary trials to maximize the efficacy of injections; in the experimental conditions highest speed was reached with the addition of 1.2 % acetic acid to the aqueous solution and making treatments in early morning or late afternoon. Considering the results of *in planta* trial, thiabendazole and allicin significantly slowed down the growth of the necroses in the growing season, in contrast propiconazole injections were impracticable.

The studies in chapters IV and V recall the methodologies applied to ash dieback, with application to chestnut ink disease complex. In particular, in chapter IV fuzzy logic theory was applied considering the environmental variables, such as minimum winter temperature, summer drought, slope's aspect, streams' distance and soil's permeability, that mainly can influence the development of the disease. The model was validated with a broad field survey conducted in a chestnut area in Treviso province. Moreover, uncertainty maps (regarding model structure, inputs and parameters) were produced for the correct interpretation of the prediction. Great part of the chestnut area in the study zone resulted as suitable for the development of ink disease, whereas only the 18.8 %, corresponding to higher elevation zones, presented inferior risks.

In a second study (chapter V), a comparative efficacy trial on four potassium phosphite formulations by means of endotherapy against chestnut ink disease is

performed. *P. cinnamomi* was isolated with baiting technique from symptomatic chestnuts and was inoculated on 50 asymptomatic trees. As a result of endotherapic treatments, the unique solution that significantly slowed down necroses' growth was potassium phosphite (35 %) with an addition of 0.1 % micronutrient solution. An additional endotherapic trial was conducted in a preliminary way in the chestnut where *P. cinnamomi* was isolated, with the main aim to evaluate growth stimulation of active growing callus next to the shape flame necroses by the injected solution of potassium phosphite 70 %. In this case, results did not highlight a significant difference between treated trees and water control ones, probably for the need of longer times for older trees.

On the base of the achieved results, epidemiological modelling and endotherapic treatments, applied both to ash dieback and chestnut ink disease, can represent fundamental tools in the management of these important diseases and should be applied in an Integrated Pest Management (IPM) approach, together with appropriate cultural techniques to maximize benefits.

Sommario

Lo scopo principale della gestione delle malattie forestali è la riduzione dei danni economici, biologici ed estetici e delle perdite di biodiversità dovute alle malattie delle piante. Le molteplici strategie usate nella gestione delle malattie possono essere raggruppate in due azioni principali, la prevenzione (anche detta profilassi) e la terapia (trattamento o cura). La prevenzione è principalmente limitata dalla mancanza di conoscenza in merito all'organismo in oggetto e i suoi ospiti. I modelli matematici sono stati utilizzati per approfondire la conoscenza delle malattie delle piante con vari obiettivi. Essi offrono l'opportunità di affrontare un uso razionale delle risorse riguardo ai costosi monitoraggi e rappresentano un passo fondamentale verso misure di controllo più sostenibili. Da un punto di vista curativo, oggigiorno gli sforzi sono focalizzati allo sviluppo di concetti di gestione delle malattie che bilancino i benefici dei pesticidi con le preoccupazioni in merito ai residui che possono contaminare l'ambiente.
In questa tesi, i due principi della gestione della malattia sono stati affrontati con due casi studio: il dissecamento del frassino, causata da *Hymenoscyphus fraxineus*, che può essere considerata la più grave malattia del genere *Fraxinus* in Europa, e il mal dell'inchiostro del castagno, causata da *Phytophthora cambivora* (Petri) Buism. and *P. cinnamomi* Rands.

Nella prima parte della tesi sono state introdotte le due malattie, in modo da poterne appurare somiglianze e differenze (Capitolo I).

Successivamente, dal capitolo II al capitolo V sono descritte le prove sperimentali effettuate. In particolare, nel capitolo II è stato approntato uno studio

della nicchia ecologica di *H. fraxineus*, con la caratterizzazione di variabili ecologiche e ambientali associate a zone naturalmente infette. Tale procedura è stata effettuata tramite *Species Distribution Models* (SDM), ampiamente utilizzati in ambito ecologico e da poco tempo anche nell'ambito della patologia vegetale. La presenza del patogeno è risultata fortemente correlata a tre variabili ambientali estive, in particolare abbondanti precipitazioni, alta umidità del suolo e basse temperature, in comparazione con la media dell'area di studio. Successivamente la tecnica dell'*ensemble forecasting* è stata applicata per ottenere una predizione della distribuzione potenziale del patogeno a scala europea, considerando la distribuzione di *F. excelsior* e *F. angustifolia*, ospiti della malattia. Infine, un innovativo metodo di *network analysis* ha permesso di individuare le aree ecologicamente adatte al patogeno ma non raggiungibili con una diffusione naturale.

Nel capitolo III viene descritto uno studio condotto per valutare sei diversi fungicidi contro *H. fraxineus*. Inizialmente è stata effettuata una prova *in vitro* dei prodotti commerciali contro cinque ceppi del patogeno. Tiabendazolo, propiconazolo e allicina sono risultati i fungicidi più efficaci, con dose letale mediana più bassa, rispetto, per esempio, al principio attivo procloraz. Al contrario, il solfato di rame e i fosfiti di potassio si sono rilevati completamente inefficaci. Successivamente, i tre migliori fungicidi sono stati applicati *in planta* tramite trattamenti endoterapici su frassini maggiori inoculati al tronco con un ceppo autoctono. Tale test è stato anticipato da prove preliminari per massimizzare l'efficienza delle iniezioni; nelle condizioni stazionali e climatiche delle prove, maggiori velocità sono state raggiunte con soluzione acquosa addizionata con 1.2 % di acido acetico, effettuando i trattamenti la mattina presto o nel pomeriggio tardo. Considerando i risultati della prova *in planta*, tiabendazolo e allicina hanno rallentato in maniera significativa la crescita delle necrosi, al contrario non si è riusciti a iniettare la soluzione a base di propiconazolo.

I capitoli IV e V riprendono le metodologie applicate contro la patologia del dissecamento del frassino, applicandole al mal dell'inchiostro del castagno. In particolare nel capitolo IV, la teoria fuzzy è stata adottata nello studio del complesso del mal dell'inchiostro, includendo nella costruzione del modello variabili ambientali quali temperatura minima invernale, siccità estiva, esposizione, distanza da corsi d'acqua e permeabilità del suolo, che più possono influire sullo sviluppo della malattia. Il modello è stato validato con un'ampia ricerca sul campo condotta

nei castagneti nell'area di Treviso. Inoltre, sono state prodotte delle mappe dell'incertezza (inerenti a struttura, input e parametri del modello) per la corretta interpretazione della previsione. Buona parte dell'area a castagneto nella zona di studio si è rivelata adatta allo sviluppo del mal dell'inchiostro, mentre solo il 18.8 %, corrispondente alle aree più elevate, presentava rischi inferiori.

Un secondo studio (capitolo V) ha riguardato una prova comparativa di efficacia di quattro formulazioni di fosfiti di potassio tramite endoterapia. *P. cinnamomi* è stata isolata con la tecnica del baiting in un castagneto affetto da mal dell'inchiostro ed è stata inoculata su 50 castagni asintomatici. In seguito ai trattamenti endoterapici, l'unica soluzione che ha significativamente rallentato la crescita delle necrosi è stata quella a base di fosfiti di potassio (35 %) addizionata con 0.1 % di soluzione di micronutrienti. Un'ulteriore prova di endoterapia è stata condotta in via preliminare nel castagneto abbandonato in cui era stata isolata *P. cinnamomi*, al fine di valutare la stimolazione alla crescita del callo cicatriziale da parte della soluzione iniettata fosfiti di potassio 70 %. I risultati ottenuti in questo caso non hanno evidenziato una differenza significativa rispetto ai controlli trattati con acqua, probabilmente per una necessità di tempi più lunghi considerando piante di età maggiore.

In base ai risultati raggiunti, la modellistica epidemiologica e i trattamenti endoterapici sperimentati in merito alle patologie del dissecamento del frassino e al mal dell'inchiostro del castagno possono rappresentare degli strumenti fondamentali nella gestione integrata delle malattie considerate, da applicare insieme ad appropriate tecniche colturali per massimizzarne i benefici.

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CHAPTER I

Introduction

Fraxinus spp.

Fraxinus is one of the 24 genus belonging to *Oleaceae* family. It is considered a monophyletic group composed by ca 50 species distributed in temperate and subtropical areas in the northern hemisphere (Wallander and Albert, 2000) and characterized by pinnate compound leaves. Three species are present in Europe: *Fraxinus excelsior* L. and *F. angustifolia* Vahl belonging to subgenus *Fraxinaster* (with flowers provided with corolla with wind pollination, which appears before defoliation) and *F. ornus* L. (included in *Ornus* subgenus, characterized by flowers provided with corolla that arises soon after leaves' emission; Bernetti, 2005; FRAXIGEN, 2005).

Among the three above mentioned ashes, *F. excelsior*, the European or common ash, is the most extensively distributed one in Europe; the species is a deciduous tree growing up to 40 m tall and with a trunk up to 1 m diameter. The buds are pyramidal, from black to dark brown; the apical one has major dimensions than the lateral ones. Common ash has compound leaves with 7-15 leaflets with minutely serrate margin, with oval to lanceolate shape. Trees can be either only male or female or hermaphroditic and flowers are grouped in inflorescences. The fruits are samara type, flattened and distally winged (Pignatti, 1997; FRAXIGEN, 2005).

The growth potential for *F. excelsior* is strictly correlated to soil's features: the species needs for rich, sandy, calcareous loam soil, with pH 7-8 (Pliura and Heurtz, 2003; FRAXIGEN, 2005). Moreover, common ash does not tolerate drought's periods and it is very susceptible to late frosts, that can even provoke its death (Lupieri, 2004). Young trees can tolerate shading, in contrast older ones are heliophilous and intolerant to lateral competition (Bernetti, 2005).

Its distribution area covers great part of Europe; the species reaches Atlantic coasts, up to Scotland, Denmark and southern regions of Sweden, Norway and Finland towards the North, and Ural mountains towards the East, but it can be found also in northern Turkey and in Caucasus region; the central zones of Spain, Italy and Greece represent the southern limit (Gellini, 1975). In Italy, common ashes are comprised mainly in ash-maple woods, whose elevation limits are defined by late frosts and water stagnation (Del Favero, 2004); taking this into account, the species can reach sub-mountain and mountain heights in Alps and pre-Alps, in contrast it can be found only in the northern zone of Apennines (Bernetti, 2005).

F. excelsior wood is creamy to light brown, with annular porosity and coarse grain, and it does not present differentiated sap wood and heartwood, although it is occasionally possible to find a dark-black heartwood (black-heart; Nardi Berti, 1994). The wood is strong, durable, resilient and easily bent, making it particularly suitable for furniture and house interiors, tool handles (e.g. hammers) and sports equipment (FRAXIGEN, 2005).

F. angustifolia, commonly denominated narrow-leafed ash, is an ash with dense crown and can reach 25 m height. It can be differed from common ash by the brown hairy buds and the leaves composed by 5-7 lanceolate leaflets with the same number of teeth as the secondary veins (Pignatti, 1997). Narrow-leafed ashes are hermaphroditic and possess inflorescences of the raceme type (FRAXIGEN, 2005).
In comparison with *F. excelsior*, narrow-leaved ash well grows on rich and moist soils and tolerates temporary flooding; optimum is found for moderately sandy-clay soils with pH 5-8 (but usually 6.0-8.0) and depth between 40 and 100 cm (FRAXIGEN, 2005). *F. angustifolia* is a light-demanding species and requires precipitation between 400 and 800 mm in order to ensure a growing season from 6 to 7 months.
Its distribution reflects the Mediterranean zone; in Italy, it can reach high altitude (500-2000 m; FRAXIGEN, 2005). The species can be usually found in the remaining strips of planitial wood and on the banks of large rivers (Bernetti, 2005).
F. angustifolia is an important species for wood production, with similar properties of *F. excelsior*, although a smaller proportion of heartwood (30-56 % *contra* 52-74 %). The species has also been widely used as an ornamental tree along roads and city streets.

F. ornus, the manna ash, is a small deciduous trees, usually not more than 15 m tall (rarely up to 20 m in humid and rich soils). The buds are grey and slightly hairy. Its flowers are small, with four white petals, comprised in big inflorescences, mainly with entomophilic pollination (Pignatti, 1997; FRAXIGEN, 2005).
It is possible to find manna ashes in a limited distribution area in southern Europe, from sea level up to an elevation of ca 1500 m. It requires high air temperatures and the optimum rainfall is from 500 to 650 mm. The surface root system is extensive and requires well-drained soils.
F. ornus mainly settles down on warm south-facing slopes, generally in association with *Quercus* spp., *Castanea sativa* Mill., *Carpinus* spp., *Ostrya carpinifolia* Scop.

and *Acer* spp. (FRAXIGEN, 2005). Low temperatures are the main limiting factor, so manna ashes can be found in southern Europe (mainly Italy and Greece), up to the southern zones of Slovakia.

F. ornus possess a lighter wood than the other two above mentioned species, but it does never reach large dimensions. Although its wood has good properties, because of its moderate dimensions it has few industrial uses (i.e. tool handles construction). Traditionally, manna is collected from the bark of young trees, which was formerly used as a laxative and today it is still produced in Sicily, in the Castelbuono and Pollina areas (FRAXIGEN, 2005).

Taking into account diseases and pests of ashes, these can be summarized as follows:

- Plant species, such as *Hedera helix*, *Clematis vitalba* and *Clematis recta*;
- Animal species, e.g. *Cervus elaphus*, *Capreolus capreolus*, *Sus scrofa*, *Lepus europaeus*, *Oryctolagus cuniculus*;
- Mites, such as *Aceria fraxinivora*, *Eriophyes fraxinivorus*, that lives in the inflorescences and causes the formation of leaning galls; *Tetranychus urticae* (red spider mite);
- Nematodes, e.g. *Meloidogyne spp.* (root-knot nematodes);
- Insects, in particular: *Prociphilus fraxini*, an aphid that conducts its holocycle between the epigeous ash parts and the roots of *Abies* genus and causes aesthetic and functional damages; *Psyllopsis fraxini*, a jumping plant lice that induces folding of the edges of leaves turning into brown galls; *Tomostethus melanopygus*, an *Hymenoptera* whose larvae feed on leaves, leaving only the ribs; *Lytta vesicatoria*, with a similar behavior, which can lead to the whole defoliation of ashes; *Lithocolletis fraxinella*, a micro *Lepidoptera* leaf miner, that causes the typical leaf miner damage with ovoid or elongated shape; *Caloptilia* (= *Xanthospilapteryx*) *syringella*, that provokes only aesthetical damages; *Dasineura fraxini*, a gall midge that causes a gall to form on the underside of the main vein; *Stereonychus* (= *Cionus*) *fraxini*, leading to moderate damages with intense and repeated ashes defoliations during the growing seasons; bark beetles such as *Leperisinus* (*Hylesinus*) *fraxini*, *Leperisinus* (*Hylesinus*) *orni*, *Hylesinus crenatus*, *Hylesinus oleiperda*; *Phloeotribus scarabaeoides*; *Chionaspis salicis*; *Eleucanium sp., Parthenolecanium sp.*; *Cossus cossus*; *Zeuzera pyrina*;

Hyphantria cunea; *Lymantria dispar*; *Euproctis chysorrhoea*; *Cerambyx cerdo*; *Aegeria aliformi*; *Operophthera brumata*;

- Bacteria: *Pseudomonas syringae* ss. *savastanoi*, causing the bacterial canker of ashes branches. The pathogen penetrates the plant through wounds, attacks parenchymatic tissues and proliferates through sap flow, originating new cancerous formations;
- Fungi: *Phyllactinia guttata* and *Mycosphaerella fraxini* can cause defoliation; *Venturia fraxini* provokes wilting and leaf fall; *Nectria cinnabarina* and *N. galligena*, *Sphaeropsis spp.*, *Armillaria spp.*, *Rosellinia necatrix*; *Heterobasidion annosum, Ganoderma lucidum, G. applanatum Phytophthora cactorum, Fomes fomentarius, Polyporus sulphureus, Coriolus versicolor, Schizophyllum* spp.; *Verticillium albo-atrum*, *Gloeosporium* spp. and *Apiognomonia* spp. (Stergulc and Frigimelica, 1996; Ferrari et al., 1999).

Ash dieback

Ash dieback is a serious disease in Europe causing death of European ash. The disease is caused by the ascomycete *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya, comb. nov. (basionym *Chalara fraxinea* Kowalski, synonym *Hymenoscyphus pseudoalbidus* Queloz et al.; Kowalski, 2006; Queloz et al., 2010; Pautasso et al., 2013; Baral et al., 2014; Pautasso et al., 2014), most likely introduced from East Asia (Zhao et al., 2012). The disease was first observed on *Fraxinus excelsior* L. in northeastern Poland in the 1990s (Przybył, 2002), but the pathogen was identified as the primary causal agent of ash dieback in 2006 (Kowalski, 2006). Symptoms were also observed in both European (*F. angustifolia* Vahl. and, only under artificial conditions, *F. ornus* L.) and introduced ash species (*F. nigra* Marsh., *F. pennsylvanica* Marsh., *F. americana* L. and *F. mandschurica* Rupr.; Drenkhan and Hanso, 2010; Kirisits et al., 2010; Webber and Hendry, 2012; Gross et al., 2014a). All age classes are affected, resulting in terminal decline.

The symptoms include necrotic leaf spots; wilting of leaves and young shoots; premature shedding of leaves; crown dieback; and necrotic bark lesions (Schumacher et al., 2010; Dal Maso et al., 2012). Below the bark, the fungus grows rapidly in the pith, paratracheal parenchyma and parenchymatic rays and phloem (Schumacher et al., 2010). Mycelium can pass through the simple pits between the parenchymatic cells and fibres (Dal Maso et al., 2012). *H. fraxineus* can colonize

wood tissues in the three dimensions and ash trees can react with the production of epicormic shoots, assuming a bushy appearance. In young stands, dieback causes major problems for establishment and regeneration (Lygis et al., 2014), whereas older trees show a slower, chronic process (Keßler et al., 2012). The presence of *H. fraxineus* was ascertained in leaves, stems, buds and roots (Przybył, 2002; Kowalski, 2006; Schumacher et al., 2007; Kirisits and Halmschlager, 2008). Recently, Cleary et al. (2012) also found the fungus inside seeds directly collected from a symptomatic tree, with important economic implications.

From an historical point of view, ash dieback was initially associated to *C. fraxinea* (Kowalski, 2006). At a later stage the fungus was initially associated as anamorph to the teleomorph *H. albidus* (subclass *Leotiomycetidae*, order *Helotiales*, family *Helotiaceae*). *H. albidus* is a saprotroph discomycete, known as *Peziza* (*Phialea cyathoidea*) *albida* Rob. already from 1851 and widely distributed in Europe. It is a colonizator specific of ash leaves rachises in the litter, where it produces white-cream apothecia in the year following the fall, that release ascospores with 1-2 septa (Desmazières, 1850; Kirisits and Cech, 2009; Kowalski and Holdenrieder, 2009). Further molecular analyses identified the teleomorph of *C. fraxinea* in *Hymenoscyphus pseudoalbidus* sp. nov. (Queloz et al., 2010). The two *Hymenoscyphus* spp. were denominated "cryptic" because they were considered morphologically identical but they presented differences in DNA sequences of *internal transcribed spacers* (ITS), calmodulin gene and elongation factor 1-α. Recently, this concept was evolved considering the presence (for *H. pseudoalbidus*) or absence (for *H. albidus*) of croziers at the ascus (Figure 1) base, strictly correlated with molecular characteristics (Baral and Bemmann, 2014). In contrast to *H. albidus*, the pathogenicity of *H. pseudoalbidus* was proved (McKinney et al., 2012). Moreover, the same authors found that *H. pseudoalbidus* is heterothallic, in contrast *H. albidus* is homothallic. This was confirmed by the absence of phialophora production by *H. albidus* colonies *in vitro* (Kirisits et al., 2013). The heterothallism of *H. pseudoalbidus* can partly explain the genetic variability among different strains from different elevations zones (Kraj and Kowalski, 2013) and the vegetative incompatibility *in vitro* among pathogen strains obtained from different *F. excelsior* trees in England (Brasier and Webber, 2013). Considering the new rules for pleomorphic fungi naming system dictated by the International Code of Nomenclature for algae, fungi, and plants, *C. fraxinea* and *H. pseudoalbidus* names

were combined by Baral et al. (2014) into:

Hymenoscyphus fraxineus (T. Kowalski) Baral, Queloz, Hosoya, comb. nov**.**

Basionym: *Chalara fraxinea* T. Kowalski, For. Path. 36: 265 (2006).

Synonym: *Hymenoscyphus pseudoalbidus* Queloz et al., For. Path. 41: 140 (2011).

The European outbreak of the disease was probably due to the introduction just once of at least two *H. fraxineus* individuals with compatible mating types from an Asian region (Gross et al., 2014b). This in accordance with Zhao et al. (2012), who reported the presence of *H. fraxineus* in Japan, recorded in 1993 under the name *Lambertella albida* (a synonym of *H. albidus*), associated with *Fraxinus mandshurica*. In contrast to *F. excelsior* in Europe, no disease symptoms caused by *H. fraxineus* have been reported on *F. mandschurica* in Japan. Furthermore, the presence of *H. fraxineus* has been recorded in China and Korea, with a slightly higher variation in gene sequences in comparison to the European reference (Zheng and Zhuang, 2013; Han et al., 2014). European populations of *H. fraxineus* have gone through a significant bottleneck during the invasion of the continent (Gross et al., 2014b), but this was probably sufficient for the replacement of the native *H. albidus* from its ecological niche (Mckinney et al., 2012). In general. invasive pathogens are often predicted to have higher pathogenicity and infectivity than native pathogens, which, on the contrary, have co-evolved with the same host for a long time, in the presence of favorable environmental conditions (Garbelotto et al., 2010).

An hypothesis of *H. fraxineus* biological cycle was proposed by Gross et al. (2012; Figure 2). In autumn infected leaves fall to the ground and the fungus winters inside, protected by the characteristic pseudosclerotial black layer that it

Figure 1. Apotecia of *H. fraxineus* on ash rachisis (Dal Maso E.).

sets up on the surface (Kowalski and Holdenrieder, 2009; Kirisits et al., 2013). In the study conducted by Gross and Holderieder (2013) it was possible to isolate the fungus from these structures also after 92 days of drying, therefore it was possible to hypothesize that they can endure at least 2 years in nature. The main hypothesis of the cycle is that the mucilaginous conidia of *H. fraxineus* could be produced in autumn at low temperature in droplets from the phialophores (Kowalski, 2006) and could act as *spermatia* (*conidiospora*), moved by water till the fusion with the *ascogonia* produced by a different mating type (Gross et al., 2012). Apotecia (Figure 1) are generated in summer on pseudosclerotial layer on the rachises remnants in the litter and release ascospores into the air mainly from half of July to half of August, early in the morning, probably because their maturation takes place in the night when the humidity is high (Timmermann et al., 2011; Hietala et al., 2013). Ascospores' infection were first observed two weeks after discharge. They can germinate on ash leaflets and rachises, develop germ tubes, followed with the production of an *appressorium* and with subsequent penetration of cuticle and epidermal cells (Cleary et al., 2013). More than one infection can occur in the same leaf, as verified by Gross et al. (2012). In the leaflets the mycelium colonize epidermal, palisade and spongy mesophyll cells and the vascular bundles (Cleary et al., 2013). Moreover, *H. fraxineus* mycelium can, at least under experimental conditions, infect through the intact, epidermis of current-year shoots of common ash and penetrate through leaf scars (Kräutler et al., 2015). After the infection, the fungus can develop inside the stem, spreading into the phloem below the bark, into the parenchymatic rays and into the xylem (Dal Maso et al., 2012), causing the characteristic cankers and dieback. The cycle is closed with the fall of leaves in autumn.

Some strains of *H. fraxineus* can produce secondary metabolites. The most important are viridin and its derivative, viridiol; the first one is phytotoxic and its activity was verified on *Fraxinus* seedlings, the second one has fungistatic and antibiotic properties (Andersson et al., 2009; Grad et al., 2009). According to Junker et al. (2013), not all the tested *H. fraxineus* strains produce viridiol *in vitro* and the concentration of the compound is not directly correlated to the pathogen's virulence. Moreover, other furanosteroids with structure similar to viridin were isolated from the pure culture, but these compounds, in contrast, do not cause similar phytotoxic effects on treated seedlings (Andersson et al., 2012).

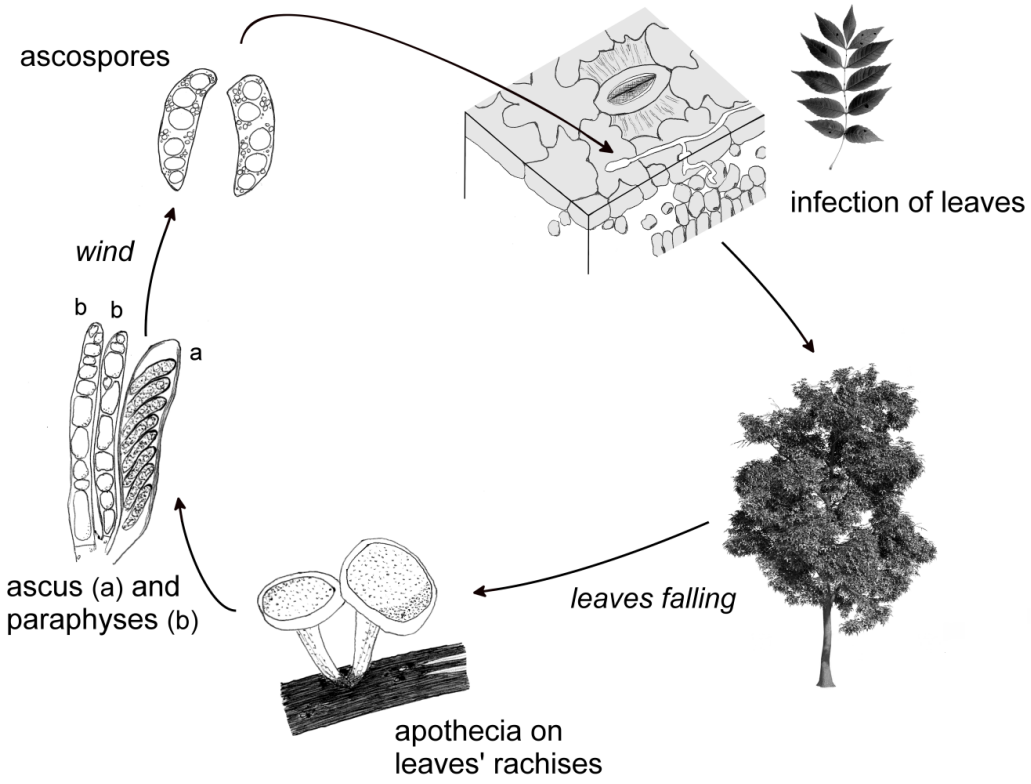

Figure 2. Hypothetical biological cycle of *H. fraxineus*.

Pham et al. (2013) extracted from pure culture mycelium other types of secondary metabolites, denominated *chalarafraxinines*, whose concentration is highly correlated to the degree of the disease in plants, and they optimized a technique for the rapid identification *in vitro* and *in vivo* of the pathogen by means of mass spectroscopy. Halecker et al. (2014) gave the name *hymenosetin* to a 3-decalinoyltetramic acid antibiotic isolated from cultures of *H. fraxineus*; the metabolite exhibited broad spectrum antibacterial and antifungal activities but was inactive in assays for evaluation of phytotoxicity, so it could constitute a defense metabolite. Moreover, *H. fraxineus* produces secondary volatiles metabolites, in particular lactones; among the four ones identified by Citron et al. (2014), 3,4-dimethylpentan-4-olide was found to be a strong germination inhibitor for ash seeds, causing necroses in the plant tissue.

Ash dieback was initially identified in Poland in 2006 (Kowalski, 2006). Currently, the disease is present in Austria, Belarus, Belgium, Croatia, the Czech Republic, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, Lithuania, the Netherlands, Norway, Romania, Russia, Slovakia, Slovenia, Sweden,

Switzerland and United Kingdom; recently, symptoms were detected also in Croatia, Romania, Russia (Kaliningrad region) and Slovakia (EPPO 2010; Ogris et al. 2009; Timmermann et al. 2011; EPPO, 2014). Due to the severity of the disease, in 2007, the fungus was added to the EPPO (European and Mediterranean Plant Protection Organization, 2007) Alert List and the NAPPO (North American Plant Protection Organization) Phytosanitary Alert List (2009); *H. fraxineus* was later deleted from EPPO Alert List because sufficient alert was considered to have been given (EPPO, 2014). Trade and consequent transport of ash logs infected by *H. fraxineus* can represent a serious threat for disease free areas. Many authors suggested phytosanitary measures such as fumigation, that was used for instance for treating oak logs against *Ceratocystis* fagacearum (Bretz) J. Hunt. At last, Hauptman et al. (2013) studied the survival of the pathogen varying moderate high temperatures and concluded that a single treatment in water at 36°C for 10 hours of ash seedlings infected by *H. fraxineus* annulled the probability of isolation of the pathogen without impairing seedlings vitality. Biosecurity protocols on disinfection were proposed to control the disease; in particular, Cooke et al. (2013) proposed various physical and chemical methods to restrict the production and spread of ascospores, including the removal of plant debris from infected sites, preventing movement of infected plant material to new sites, the use of disinfectants to treat contaminated footwear, clothing and equipment and the use of fungicides and biocides for the treatment of infected debris. Until now information of fungicides effective against the disease is not complete (Cooke et al. (2013)), but the potential for a cure was considered to be high with carbendazim, being able to stop the production of apothecia after fungicidal treatments (Hauptman et al., 2012). These authors found, moreover, effective concentrations of urea to inhibit *in vitro* growth of *H. fraxineus* and formation of its apothecia, but attention should be paid because low concentration could even enhance the formation of apothecia or positively affect disk area of the developed apothecia (Hauptman et al., 2014). Moreover, genetic studies on the resistance toward *H. fraxineus* were conducted with good results on different clones of *F. excelsior* and permitted to distinguish clones with different susceptibility (Pliura et al,. 2011; Kjaer et al., 2012; McKinney et al., 2012; Stener, 2013; Cleary et al., 2014; Pliura et al,. 2014). Although completely resistant ashes have not been identified yet, identification and propagation of highly superior ash trees offers a potential route to revitalize and

restore ash forests in the future (McKinney et al., 2014).

In 2012, the FRAXBACK COST Action was specifically constituted for a period of 4 years, in order to share the knowledge on the disease and elaborate guidelines for a sustainable management of ashes in Europe.

Castanea sativa Mill.

Castanea genus, belonging to *Fagaceae* family, is composed by a variety of deciduous trees and shrubs. Most common cultivated species are *Castanea crenata* Sieb and Zucc. (the Japanese chestnut), *C. dentata* (Marsh.) Borkh. (the American chestnut), *C. mollissima* Bl. (the Chinese chestnut) and *C. sativa* Mill. (the sweet chestnut; MacDonald, 1993). In Italy, sweet chestnut has been cultivated since antiquity and find its optimum in the *Castanetum* area (400 and 1000 m in altitude). The species is exothermic and suffers winter temperature inferior to -15 °C and summer droughts (Lazzara et al., 1990; Borin et al., 2003).

The sweet chestnut is a deciduous and long-lived species, with 15-20 m average height (exceptionally 30-35 m) and a root system robust but little developed in depth (Paglietta and Bounos, 1979; Bravo, 1949). It prefers acid, siliceous and volcanic soils, with pH between 4.5 and 6.7, with high permeability and organic matter content (Lazzara et al., 1990; Bassi, 2008). Minimum rainfall is 800 mm and main limitation for nut production is low temperatures, which enable the female flowers to be fecundated and to develop a normal kernel (Breisch, 1995).

Chestnut has been classically described as a monoecious species, with flowers of both sexs arising from the leaf axis of current season's growth; male flowers present stamens up to 35 cm in length and meiosis occurs in the first week of June in Italian cultivars, 10-15 days before anthesis (Botta et al., 1995); female flowers are grouped in 3-5 inflorescences and every flower presents generally 7 carpels and could have stamen remnants on the base of the style (Pereira-Lorenzo and Ramos-Cabrer, 2004). Pollen is wind-dispersed and cross-pollination is essential for normal production (Breisch, 1995; Soylu et al., 1999). After the fecundation, a thorny husk envelops the fruit (Borin et al., 2003).

New orchards should be established on slopes to take advantage of the good drainage (Pereira-Lorenzo and Ramos-Cabrer, 2004); on the contrary, convex shaping of land is suggested on plain plot (Bassi, 2008). Chestnut wood for nuts production is usually governed as high forest, with low density (100-150 trees/ha);

commonly, graft is made in the field after four years (Pereira-Lorenzo and Ramos-Cabrer, 2004). Orchards for timber and nut production can be also maintained as coppice stand, with a much higher density than the nut-only orchards (7 × 7 m). Orchards' management consists of pruning (every 5 years), fertilization and removal of suckers at the collar (Lazzara et al., 1990).

Chestnuts are affected by a conspicuous group of pathogens. Since the first observation in Genova in 1938, *Cryphonectria parasitica* (Murr.) Barr causing canker blight, has spread quickly in Italy and Europe. The fungus destroys the bark and the cambium causing the death of distal branches or of the entire tree when girdling around the trunk (Pereira-Lorenzo and Ramos-Cabrer, 2004; Vettraino et al., 2008). Biological control is practiced applying hypovirulent strains according to Grente's method (Grente and Berthelay-Sauret, 1978). Other parasites can be responsible of chestnuts' decline (MacDonald, 1993); in particular root rot can be caused by *Armillaria* spp. Overall, serious root damages can be provoked by *Phytophthora* species associated to ink disease (see next section for further information; Vettraino et al., 2008).

Two chestnut weevils (*Cydia splendana* Hubner and *Curculio elephas* Gyll.) and two fungi (*Phoma endogena* Speg. and *Rachodiella castaneae* Pyr.) were the main responsible of nut losses (Pereira-Lorenzo and Ramos-Cabrer, 2004). Since the first observation in Italy in 2002, the gall wasp *Dryocosmus kuriphilus* Yasumatsu has spread both by active flight of adults and together with the transport of infected material. The pest, originated from China, attacks chestnuts spring shoots and buds, inducing severe plant decline and drastic yield reductions (Graziosi and Santi, 2008). Trials of biological control started in Italy with the introduction of the parasitoid *Torymus sinensis* Kamijo from China's mainland, with encouraging results (Quacchia et al., 2008; Sartor et al., 2009).

C. sativa is a multipurpose tree valued for its nuts, timber, secondary products (i.e. tannins, honey, associated fungi), forest landscape and biodiversity (Fenaroli, 1945; Malandrino and Gruppo lavorazioni forestali dell'assolegno, 1983; Lazzara et al., 1990; Pereira-Lorenzo and Ramos-Cabrer, 2004). This traditional crop has declined in importance due to diseases and low profitability, especially in old plantations, together with the abandonment of mountainous areas (Malandrino and Gruppo lavorazioni forestali dell'assolegno, 1983; Pereira-Lorenzo and Ramos-Cabrer, 2004; Buonous, 2005). The species is a long-term investment where returns

are limited but nowadays its stable fresh market and the need of life suitability for mountain people encourage the growers to establish new plantations and recover old orchards (Pereira-Lorenzo and Ramos-Cabrer, 2004; Turchetti and Maresi, 2008; Beccaro et al., 2009; Blom et al., 2009; Costa et al., 2011).

Chestnut ink disease

Chestnut ink disease (CID) is one of the most destructive diseases of sweet chestnut (Vannini and Vettraino, 2001; Vettraino et al., 2005; Choupina et al., 2014). CID is known since 1917 (Petri, 1917, 1923) and is increasingly spreading in many European chestnut forests and plantations (Turchetti and Maresi, 2008; Vettraino et al., 2008; Beccaro et al., 2009; Costa et al., 2011; Woodward et al., 2011). The disease is caused by two soil-borne pathogens *Phytophthora cambivora* (Petri) Buism. and *P. cinnamomi* Rands; other *Phytophthora* species were found associated with chestnut root system, but their role could be limited to fine roots' damage (Vettraino et al., 2005).

Accordingly to recent taxonomy, *Phytophthora* genus, comprising over 100 recognized species (Kroon et al., 2012) and initially included in the *Fungi* kingdom (Petri, 1917), belongs today to kingdom *Chromista* (Smith, 1988; Hawksworth et al., 1995; Agrios, 2005), in particular:
Kingdom *Chromista*; Phylum *Oomycota*; Class *Oomycetes*; Order *Peronosporales*; Family *Pythiaceae*; Genus *Phytophthora*

The epidemiology associated with *Phytophthora* is considered multicyclic (Erwin and Ribeiro, 1996). The mycelium of *P. cinnamomi* can produce three type of vegetative fructifications: chlamydospores (average diameter 41 µm; Figure 3), sporangia (average 75 x 45 µm; Figure 3), within 20-30 natant zoospores are produced (25-35 mm; Erwin and Ribeiro, 1996). These spores can move on short distances attracted by chemical and electrical signals emitted by the apex of growing plant roots (Gara et al., 2005; Deacon, 2000). When a zoospore encounters a root, it loses the whips, encysts and adheres to the surface of the root by means of an adhesive substance produced by the cellular wall; subsequently, a germ tube penetrates the root apex and the mycelium can develop inside the host (Figure 4; Gara et al., 2005).

P. cinnamomi is heterothallic. Sex organs, the oospora (size range 19 - 54 µm, depending on the medium), are produced by sexual recombination of A1 and A2

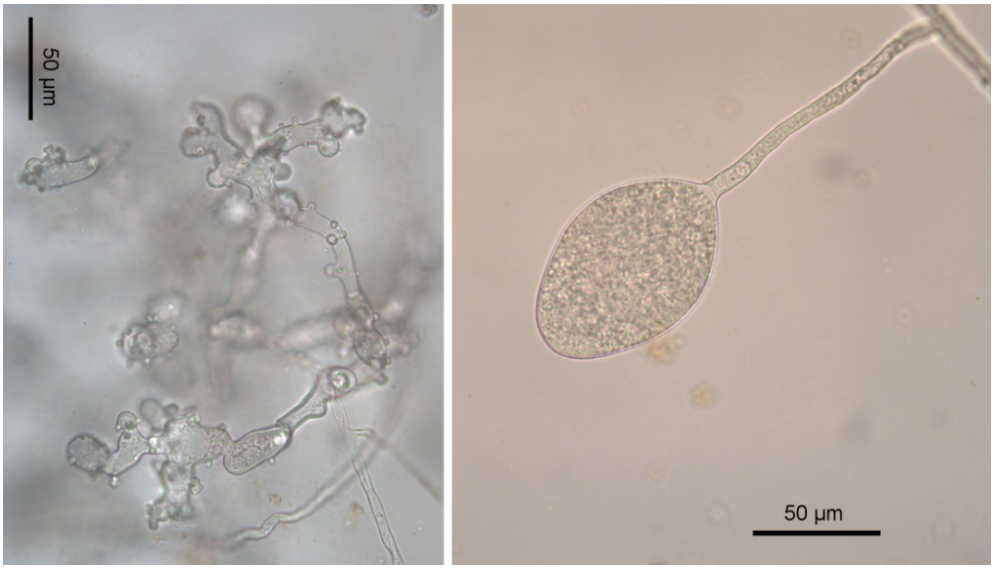

Figure 3. *P. cinnamomi* under the microscope. On the left, the coralloid mycelium; on the right, a typical sporangium (Dal Maso E.).

generations of the pathogen (Figure 4). *Phytophthora cambivora* is morphologically similar to *P. cinnamomi* and they are considered a unique taxon with different genetic structures but unique behavior (Brasier, 1999).

C. sativa affected by CID exhibits reduced leaf size, dieback of the distal branches, canopy dieback, cracked areas at the base, root and collar necroses, bark detachment, tannic fluid leaks, gradual decline and host death (ANNEX 1; Vannini and Vettraino 2001; Vettraino et al. 2005; Vannini et al., 2010; Prospero et al., 2013). Disease progression can be slow, leading chestnuts to a chronic dieback, or rapid, killing also large trees in few growing seasons (Jung et al., 2000; Balci and Halmschlager, 2003; Jung et al., 2005). The loss of chestnuts entailed not only an economical and cultural damage, but it compromises also the stability of slopes or ridges, leaving them exposed to erosion from runoff rainwater (ANNEX 1; Maresi and Turchetti, 2008).

CID was discovered in Portugal for the first time in 1838 on *C. sativa* (Crandall et al., 1945), but probably was already present in Spain since 1726 (Crandall, 1950). In Italy Gibelli observed the characteristic symptoms in 1875, but he did not identified the pathogen. The species was described for the first time as *Blepharospora cambivora* by Petri (1917). In 1923 the Italian Government issued a "Decreto ministeriale di Lotta Obbligatoria", then abrogated in 1998.

High mortality caused by CID have been observed in various areas of Europe

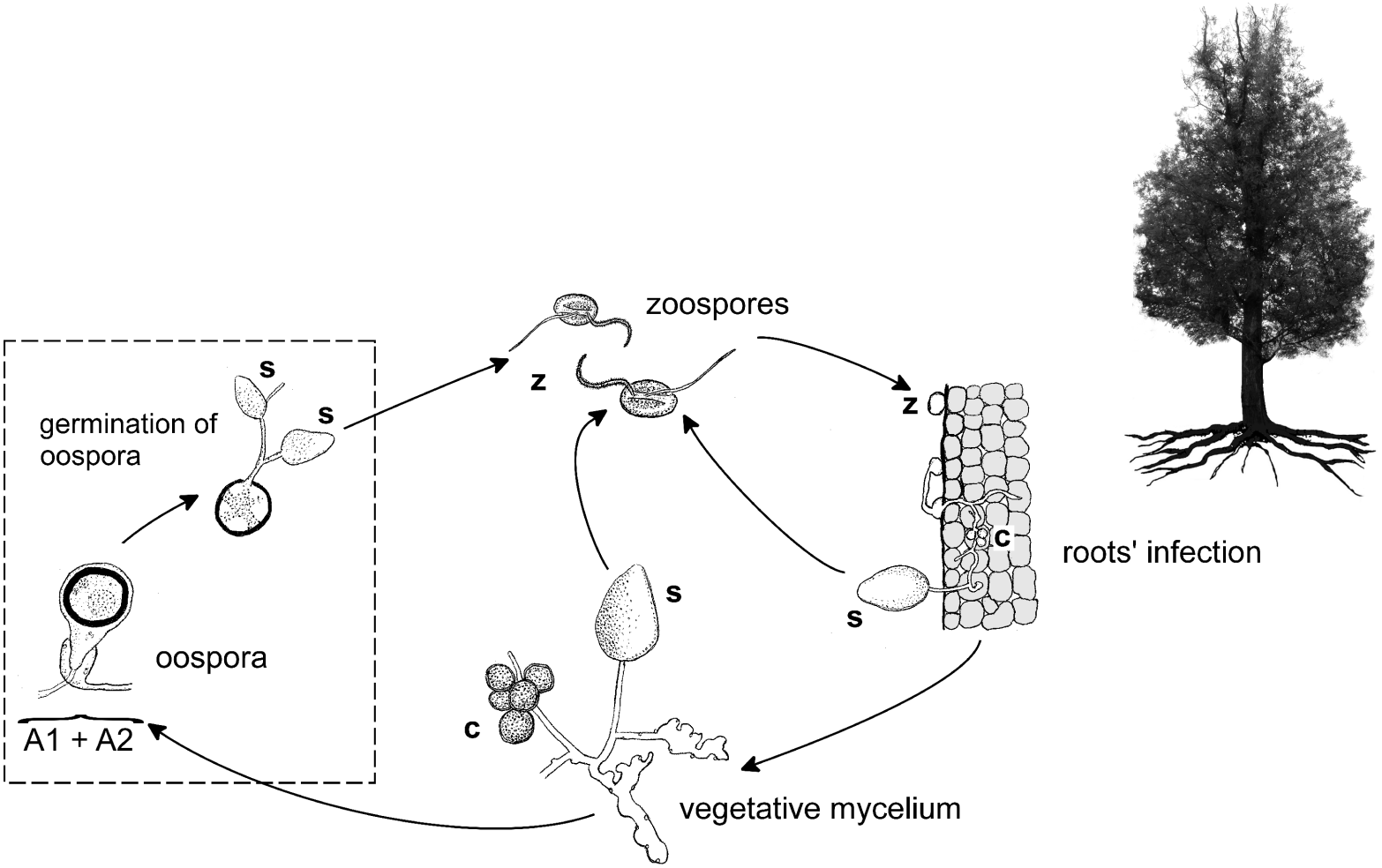

Figure 4. Synthetic representation of *P. cinnamomi* life cycle. The dotted box contains the sexual phase, where A1 and A2 represent the two mating types. Abbreviations: c = clamydospores; s = sporangium, z = zoospores.

and USA (Anselmi et al., 1996; Olsen, 2000). The soil-borne pathogens are animals and waterways dependent for long-range dispersal (Vannini et al., 2010; Gonthier and Nicolotti, 2013), while they can disperse actively for little distances as natant zoospores (Brasier, 1999).

CID can be prevented or controlled by integrated chemical and agronomic measures and protocols, that imply, for instance, management of water's flows and fertilizing (IPC; Bounous and Abreu, 1998; Brasier, 1999; Gentile et al., 2010).

Hybridization was proposed among the first solutions against CID and there were various attempts to obtain resistant varieties that were also vigorous and productive, with contrasting results considering acclimatization, adaptability to different environment and fruits production (Gomes-Pereira et al., 1993; Bounous and Abreu, 1998; Craddock and Bassi, 1999; Guedes-Lafargue and Salesses, 1999; Bounous and Beccaro, 2004).

Among compounds employed in CID chemical control, copper compounds represent efficient solutions against *Phytophthora* spp. First application can be assigned to the "Gandolfo" method, consisting in pouring bordeaux mixture 5 % additioned with copper oxychloride on the trunk base and main roots, then exposed to winter temperature (Paglietta and Bounos, 1979; Vannini and Vettraino, 2001). As disadvantages, copper compounds were proved to deliver toxic impact on micobiont when applied to the soil (Graham et al., 1986).

Among systemic compounds, some phenylamide fungicides are indicated as effective against *Phytophthora*. In particular, *P. cinnamomi* growth is highly sensitive to the water soluble metalaxyl (Erwin and Ribeiro, 1996), so that the compound was suggested together with copper sulphate for soils treatment. However, its use should be carefully considered, as it can entail the insurgence of resistant isolates (Franceschini, 2011).

Phosphonates are effectives both *in vitro* and *in planta* against *P. cinnamomi* and *P. cambivora* (Coelho et al., 2005; Hardy et al., 2001; Wilkinson et al., 2001; Gouveia et al., 2010), acting directly at high concentration or stimulating host defence at low concentration (Jackson et al., 2000). In comparison with phosphite foliar treatments (Pilbeam et al., 2000; Hardy et al., 2001), trunk injection can lead to less or none phytotoxic effect, varying considerably with the dose and at a family and genus level (Garbelotto et al., 2007). Phosphite trunk injections was proved effective against *Phytophthora cinnamomi* in Avocado and in chestnuts

(Darvas et al., 1984; Gentile et al., 2009), but little is known about ideal concentration and potential adjuvants.

Moreover, the positive role of ectomycorrhizae (EMs) in controlling *P. cambivora* and *P. cinnamomi* infections in chestnut has been demonstrated both in laboratory and greenhouse trials on seedlings and micropropagated plants (Branzanti and Zambonelli, 1986; Branzanti et al., 1994; Martins et al., 1996, 1997; Branzanti et al., 1999). At forest level, ectomycorrhizal community composition and abundance can be used as bioindicators of early and asymptomatic stages of the disease (Scattolin et al., 2012; Corcobado et al., 2014).

CHAPTER II

Risk of natural spread of *Hymenoscyphus fraxineus* with environmental niche modelling and ensemble forecasting technique

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Abstract

Ash dieback, caused by the ascomycete *Hymenoscyphus fraxineus*, is rapidly expanding over large geographical areas in Europe. A myriad of factors influence pest invasions and long-term establishment, i.e., species' life stage, the availability of suitable hosts and the suitability of the environment. This paper examines the principal environmental features that characterise naturally infected zones in order to forecast the potential distribution of the pathogen within the ranges of European ash species by means of Species Distribution Modelling and an ensemble forecasting technique. Furthermore, a network analysis permitted dispersal dynamics to be included in order to obtain realistic risk predictions for the natural spread. The multi-modelling procedure allowed the most endangered regions to be identified as the central and eastern Alps, Baltic States, southern Finland and the area encompassing Slovakia and southern Poland, whereas most marginal regions of the study area appeared less suitable for the natural establishment and spread of the disease. Statistical model predictions were highly correlated with abundant summer precipitation, high soil moisture and low air temperature. A novel approach to the ensemble forecasting technique in epidemiological modelling of plant pathogens is suggested as a tool to aid the survey of this infectious disease. Moreover, the final potential distribution maps may promote discussions about the control of the disease and the risks associated in the trade or movement of ash species.

Keywords

ash dieback; *Hymenoscyphus pseudoalbidus*, *Chalara fraxinea*; *Fraxinus*; Species Distribution Models; epidemiology.

Abbreviations

GDD, Growing Degree Days; TSS, True Skill Statistic; AUC, area under the curve; ROC, Receiver Operating Characteristic; GLM, Generalised Linear Model; LOG, Logistic Regression Model; SVM, Support Vector Machine Model; MLP, Multilayer Perceptron Artificial Neural Network; CHAID, Chi-squared Automatic Interaction Detector Classification Tree; WA, weighted average.

Introduction

Ash trees in Europe are threatened by a major disease caused by the ascomycete *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya, comb. nov.

(basionym *Chalara fraxinea* T. Kowalski, synonym *Hymenoscyphus pseudoalbidus* Queloz et al.; Kowalski, 2006; Queloz et al., 2010; Pautasso et al., 2013; Baral et al., 2014), most likely introduced from East Asia (Zhao et al., 2012). The disease was first observed on *Fraxinus excelsior* L. in northeastern Poland in the 1990s (Przybył, 2002), but the pathogen was identified as the primary causal agent of ash dieback in 2006 (Kowalski, 2006). Symptoms were also observed in both European (*F. angustifolia* Vahl. and, only under artificial conditions, *F. ornus* L.) and introduced ash species (*F. nigra* Marsh., *F. pennsylvanica* Marsh., *F. americana* L. and *F. mandschurica* Rupr.; Drenkhan and Hanso, 2010; Kirisits et al., 2010; Webber and Hendry, 2012; Gross et al., 2014a).

Wind-dispersed ascospores, produced during the summer in apothecia on the previous year's leaf remnants in the litter, can penetrate and infect ash leaves via appressoria (Timmermann et al., 2011; Cleary et al., 2013; Gross et al., 2014a). The symptoms that subsequently develop include necrotic leaf spots; wilting of leaves and young shoots; premature shedding of leaves; crown dieback; and necrotic bark lesions extending to the phloem, paratracheal parenchyma and parenchymatic rays below the bark (Schumacher et al., 2010; Dal Maso et al., 2012).

At the present time, fully effective measures to control the disease are still lacking (Cooke et al., 2013; Hauptman et al., 2013, 2014). Due to its rapid spread in the majority of European countries (Kowalski, 2006; Timmermann et al., 2011; Sansford, 2013), *H. fraxineus* was added to the EPPO Alert List in 2007 but was later deleted because sufficient alert was considered to have been given (EPPO, 2014).

Predicting the spread of emerging infectious diseases is fundamental for forecasting potential ecological consequences and designing control strategies (Paul and Munkvold, 2005; Dupin et al., 2011; Meetmeyer et al., 2011; Jönsson and Thor, 2012; Santini et al., 2013; Lõhmus and Runnel, 2014), and mathematical models have long been widely used for agricultural and forest diseases (ANNEX 2; Paul and Munkvold, 2005; Dupin et al., 2011; Van Maanen and Xu, 2003; Meentmeyer et al., 2004; Bergot et al., 2004; Auclair et al., 2010), in particular, to predict the spread of parasites and pests (Sturrock et al., 2011; Venette and Cohen, 2006; Klopfenstein et al., 2009; Pukkala et al., 2005; BenDor et al., 2006) or the risk of infection in pest-free areas (Kelly et al., 2007; Ganley et al., 2009; Robinet et al., 2012). Among those extensively employed, Species Distribution Models (SDMs) can

identify statistical or logical functions linking species' occurrences to a series of predictors, and project these relationships onto a geographical space, allowing range dynamics to be estimated and *suitability maps* defined (Kamino et al., 2012). Infact, the SDM approach is based on the concept of Grinnellian niche as a constraint to the potential distribution of species and can easily be implemented using ecological and evolutionary assumptions (i.e., selecting the most causal environmental predictors or determining a restricted set of competing models in multi-model inference; Peterson, 2003; Guisan and Thuiller, 2005).

Some environmental characteristics connected to a pathogen's biology are known or hypothesised from field observations and laboratory experimental proofs. In terms of temperature requirements, *H. fraxineus* can be classified as a mesophile (Hietala et al., 2013), considering that most isolates in pure culture show their maximal growth rate at approximately 20°C and cease to develop at approximately 30°C (Kowalski and Bartnik, 2010). However, in ash tissues, the fungus exhibits less tolerance to heat (Hauptman et al., 2013). On the other side, the pathogen is considered a cold-tolerant organism because of the ability of producing necroses during the winter and phialides at low temperature (Kowalski and Bartnik, 2010; McKinney et al., 2012). The asexual stage of the pathogen is most likely strongly associated with the pseudosclerotial plates that it produces on infected rachises (Gross et al., 2014a) and that allow the fungus to overwinter (Schumacher et al., 2010; Cleary et al., 2013). The main hypothesis for subsequent fertilisation is proposed by Gross et al. (2012) and supposes that conidiospores (spermatia), readily produced on the petiole in autumn, could be mediated by free water till the fusion with an ascogonium (Schumacher et al., 2010; Gross et al., 2012). Ascospores of *H. fraxineus*, produced in the leaf litter by apothecia, are windborne and secure the dispersal and spread of the pathogen (Bengtsson et al., 2012; Gross et al., 2012). They are produced in abundance during several months in late spring and summer and are considered drought sensitive (Gross et al., 2014a). Furthermore, Husson et al. (Husson et al., 2012) found a positive correlation between soil moisture and the percentage of affected collar circumference caused by *H. fraxineus* in northeastern France. Additionally, Gross et al. (2012) supposed that moist soil conditions could favour the survival of the pathogen on ash rachises in the litter and apothecia production. The discharge of spore has a peak in the morning (Timmermann et al., 2011), most likely to prevent spore desiccation and to

facilitate germination (Hietala et al., 2013). Moreover, depending on altitude and related climatic conditions, the pathogen's apothecia first appear at the end of May, June or early July, subsequently with a different duration of dispersal (Kräutler and Kirisits, 2012); in addition, the genetic intrapopulation variability of *H. fraxineus* is highly dependent on elevation, and, together, on the number of days with snow cover (Kraj and Kowalski, 2013).

The artificial long-distance movement of infected ash commodities is known to have contributed to the spread of the disease (Pautasso et al., 2013; Gross et al., 2014a), so that a Plant Health Order was introduced in Great Britain to restrict imports of ash plants and seeds to those originating in pest-free areas, despite the confirmed presence of the pathogen in a number of sites in the country (Elith and Leathwick, 2009), but little is known about the natural spread potential of *H. fraxineus* when considering habitat suitability. According to official reports (Timmermann et al., 2011; EPPO, 2014), not all the distribution ranges of *F. excelsior* and *F. angustifolia* are affected yet. By means of an ensemble forecasting technique, resulting from a combination of nine distribution models, the main objective of this study was to examine the potential natural distribution of the parasite in European and neighbouring regions according to the geographical distribution of its hosts and to the main environmental features of the sites in which the natural presence of the disease was reported. Secondly, the natural spread of the pathogen was simulated, in order to consider the role of airborne spores in dispersal. In this perspective, nurseries and recent plantations that may be associated with the movement of infected plants for planting (Harwood et al., 2009; Great Britain Forestry Commissioners, 2012; Pautasso et al., 2013; Gross et al., 2014a) were intentionally excluded.

Materials and Methods

Study area

The extension of the study area was based on the natural distribution maps of the three indigenous European ash species known to be susceptible to the parasite under natural conditions (Kirisits et al., 2009, 2010). *F. excelsior* and *F. angustifolia* distribution maps were obtained from EUFORGEN and FRAXIGEN official databases with previous authorisation (EUFORGEN, 2013; FRAXIGEN Research Project, 2013), imported into Quantum GIS software (QGIS Development

Team, 2013), and then converted into a single georeferenced shapefile corresponding to the study area (Figure 5). On the contrary, *F. ornus* was not included in the modelling because this species can develop limited necrotic lesions after artificial inoculation but appears to be resistant under field conditions (Pautasso et al., 2013).

Pathogen's presence

The greatest number of scientific reports of *H. fraxineus* were collected in a data-set by means of a wide bibliographic study (used keywords "*Hymenoscyphus pseudoalbidus*", "*Chalara fraxinea*", "ash dieback", "dieback" or "decline of *Fraxinus excelsior*" or "European ash" or" common ash" or "*Fraxinus angustifolia*" or " narrow-leafed ash", and the combinations of these in all languages of the countries included in the study area; time frame for the research 01/06/2013-30/09/2013; publications had to be scientific papers referring to records of the disease that were spatially included in the study area; Schumacher et al., 2007; Bakys et al., 2009; Kirisits, 2008; Jankovský and Holdenrieder, 2009; Kowaslki, 2009; EPPO, 2010; Kowalski and Czekaj, 2010; Chandelier et al., 2011; Husson et al., 2011; Kunca,

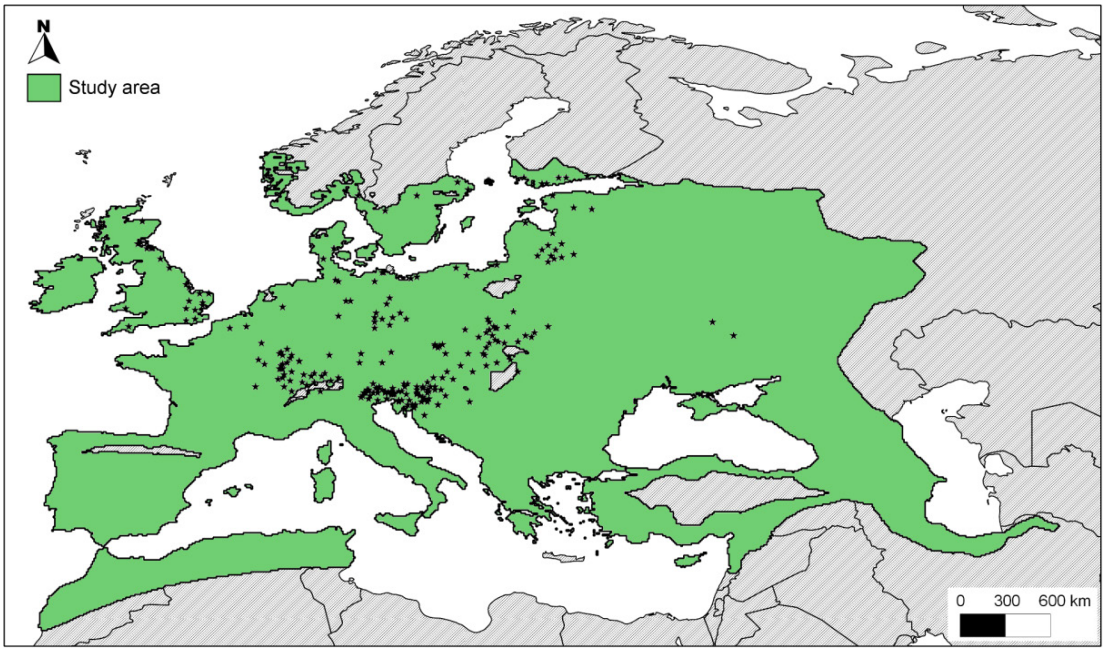

Figure 5. Study area and presences of *H. fraxineus* derived from natural infection. The area was obtained by merging the chorological maps of *F. excelsior* and *F. angustifolia*. Stars indicate the 252 localities where the presence of the pathogen was associated with a natural infection process.

2011; Rytkönen et al., 2011; Witzel and Metzler, 2011; Barić et al., 2012; Baumann et al., 2012; Conedera et al., 2012; Timmermann et al., 2011; Husson et al., 2012; Koltay et al., 2012; Lenz et al., 2012; Stenström et al., 2012; Županić et al., 2012; Bakys et al., 2013; Davydenko et al., 2013; Forestry Commission, 2013; author, unpublished data). As the movement of asymptomatic, infected plants for planting is responsible for the artificial spread of the disease (Pautasso et al., 2013; Gross et al., 2014a), reports in plantations younger than 3 years (the minimum time for confirmation that the site could be suitable for the pathogen; Varstvo gozdov Slovenije, 2013) and in nurseries were excluded from processing (16 records excluded). In this way, 252 sites with symptomatic ashes within the study area were considered (Figure 5).

This type of data can show patchy spatial coverage and some regions where the detected ash dieback had a greater recorded density than others, which was most likely derived from different sampling methods (Loiselle et al., 2008; Newbold, 2010). Moreover, these types of records are often closer to roads, rivers, coasts, towns and cities or concentrated in areas that are of more interest to collectors than they would be if the survey effort were completely random (Hijmans et al., 2000; Soberón et al., 2000; Funk and Richardson, 2002; Reddy and Davalos, 2003). To correct for this spatial bias, the resolution of the final study area was fixed in a 0.5° x 0.5° regular grid considering the spatial accuracy and precision of species records, according to Dungan et al. (2002). Presence points were then intersected with this grid, thereby reducing the number of presences to 177 patches containing at least one infected point (Farina, 2001).

Environmental variables

The predictor set included 12 environmental variables. For every predictor with a temporal scale, a subset January 1992 - December 2013 (Timmermann et al., 2011) was considered and monthly averages were computed in Raster Map Calculator in GRASS GIS (GRASS Development Team, 2013). The variables were selected for their relevance to the pathogen's biology and current main hypotheses on its life cycle, as reported in the introduction section. Precipitation, frequency of days with frost and monthly mean temperature maps were obtained from available Climatic Research Unit (CRU) time-series datasets (Mitchell and Jones, 2005; Harris et al., 2014), and the Growing Degree Days (GDD) computation was performed with a

temperature threshold of -10°C (Snyder, 1985; Hietala et al., 2013). Maximum, mean and minimum monthly temperature at a height of 2 m, surface temperature, soil moisture (0-10 cm depth) and runoff were obtained from NCEP/NCAR Reanalysis 2 (Kalnay et al., 1996; Kanamitsu et al., 2002). Snow cover and elevation maps were acquired from MODIS/Terra Snow Cover Monthly Dataset (National Operational Hydrologic Remote Sensing Center, 2004) and from SRTM 90 m Digital Elevation Data (Jarvis et al., 2008), respectively. Wind speed and direction were obtained from NCEP/NCAR Reanalysis 2 (Kalnay et al., 1996; Kanamitsu et al., 2002) but, after computing monthly averages in the considered temporal range, final maps showed no spatial pattern in the study area (Fink et al., 2010; QGIS Development Team, 2013), also for the months more suitable for spores' dispersal (Timmermann et al., 2011). Therefore, these predictors were discarded from further analyses.

To avoid multi-collinearity (Graham, 2003) and model over-fitting (Peterson et al., 2007), the 122 environmental predictors (10 monthly predictors plus digital elevation data and GDD November-March, according to Table 1) were subjected to a collinearity control, based on the Pearson correlation between predictors (Table 1; Dormann et al., 2013). In this way and according to Peers et al. (2013), when the correlation between two variables was statistically significant for $r>0.85$ and $p<0.0001$ (IBM SPSS Statistics software v. 22, International Business Machines Corp., New York, USA; IBM Corp. Released, 2013), the most adequate predictor was selected using information about the fungal biology (Dupin et al., 2011; Jopp et al., 2011; Braunisch et al., 2013). According to Merow et al. (2013), this approach eliminates correlation and allows more parsimonious and interpretable models. Finally, the resulting maps were overlaid with the grid's study area while considering the average values in the centroids (the centre points of defined areas; de Smith et al., 2007).

Pre-processing of data

The modelling was directly trained in the whole study area as no other regions with similar environmental ranges, hosts and disease's presence have been detected to date (EPPO, 2014), nor could a subplot represent all the considered climatic zones (Barve et al., 2011; Owens et al., 2013). Moreover, taking into account the unavailability of absence data for the pathogen (Václavíck and Meentemeyer,

Chosen predictor	Monthly averages calculation	Variables correlated (r>0.85, p<0.0001) and discarded
Digital elevation data		
Mean temperature	*	Maximum, minimum and mean temperature at a height of 2 m; Skin temperature
Frost day frequency	*	Snow cover
Precipitation	*	
GDD November-March		Maximum, minimum and mean temperature at a height of 2 m; Skin temperature
Soil moisture (0-10 cm depth)	*	Runoff

Table 1. Environmental variables considered in the study. The table shows the variables used for model building and the discarded predictors after the collinearity control based on the Pearson correlation (r). Stars indicate the selected predictors for which a monthly average was computed.

2009), background data (also referred as "pseudoabsences") were included in model construction (Elith et al., 2006; Chefaoui and Lobo, 2008; Barbet-Massin et al., 2012). Pseudoabsences were generated randomly, reducing the number of background points to three times the number of presences, according to Wisz and Guisan (2009) and Barbet-Massin et al. (2012). The resulting data were then separated in three partitions in a split-sample approach (IBM SPSS Statistics software v. 22, International Business Machines Corp., New York, USA; Van Houwelingen and Le Cessie, 1990; IBM Cop. Released, 2013): training (65 %, comprising 115 presences and 345 pseudoabsences) and validation (15 %, with 27 presences and 80 pseudoabsences) sets were used in the construction and calibration of the individual models with the control of overfitting (Sheriff et al., 2004), respectively. The remaining data (test set, 20 %, with 35 presences and 106 pseudoabsences) were used for comparing models (Nelles, 2001).

Species Distribution Modelling procedures

In accordance with Elith et al. (2006) and Guisan and Thuiller (2005), more than one modelling algorithm, both classical and novel, was adopted. Methods were grouped on the basis of algorithm class into the following five categories: 1)

Regression based models, 2) Classification Trees (Rokach and Maimon, 2008) and, within the machine learning community, 3) Artificial Neural Networks (ANN; Priddy and Keller, 2005), 4) Support Vector Machine (SVM; Wang, 2005) and 5) Maxent (Phillips et al., 2004, 2006). The chosen regression-based models were backward stepwise logistic regression (with only main effect or with first order interactions; LOG) and a Generalised Linear Model (GLM), considering a binomial distribution, previously used extensively in species' distribution studies (Guisan and Zimmermann, 2000; Rushton et al., 2004; Segurado and Araújo, 2004; Elith and Graham, 2009; Rupprecht et al., 2011; Zurell et al., 2011; Smith and Hoffman, 2001). CHAID (Chi-squared Automatic Interaction Detector), belonging to the category "Classification tree", was chosen in order to take advantage of multiple splitting pathways for each grid's node (Scarnati et al., 2009; Clark et al., 2012) and two models were built, considering both boosting and bagging (bootstrap aggregation) procedures (De'ath, 2007). Boosting and bagging procedures were also performed within the Artificial Neural Networks (ANN) Multilayer Perceptron category (MLP), which is considered more powerful than multiple regression models when modelling nonlinear relationships (Guisan and Zimmermann, 2000; Williams et al., 2009). The last two machine learning algorithms used were the Support Vector Machine (SVM), recently introduced in a species distribution context (Guo et al., 2005; Drake et al., 2006), and Maxent (Maximum Entropy Model), estimating the target probability distribution by finding the probability distribution of maximum entropy (Phillips et al., 2004, 2006; Clark et al., 2012). Among the five categories of model construction, 1 to 4 were built in IBM SPSS Statistics software (v. 22, International Business Machines Corp., New York, USA; IBM Corp. Released, 2013), and the SVM algorithm was implemented in LibSVM library (v. 3.17; Chih-Chung and Chih-Jen, 2011), while the Maxent model was produced in Maxent software (v 3.3.3k; Phillips et al., 2004, 2006). For each type of model, respective statistical parameters were calibrated in order to optimise the resulting sensitivity. Multiple runs (maximum = 50) for each model gave the distribution probability in each cell, which generated a final output with a mean predictive cell value ranging from 0 to 1.

Evaluation statistics

To evaluate the performances of the nine models, the predicted values were

compared with the test set by means of contingency tables (also called a confusion matrix; Li and Guo, 2013) obtained with IBM SPSS Statistics software (v. 22, International Business Machines Corp., New York, USA) and considering the conventional threshold of 0.5 (Liu et al., 2005) predicted relative probabilities ≥ 0.5 were classified as presence, whereas relative probabilities < 0.5 were classified as absence. The classical measures derived from the confusion matrix and calculated in Microsoft Excel (v. 2007, Microsoft Corporation, Redmond, USA; Microsoft, 2007) were a) overall accuracy, b) specificity, c) sensitivity, d) Kohen's Kappa statistic and e) the True Skill Statistic (TSS) (Table 2; Liu et al., 2005; Allouche et al., 2006). The Area Under the Curve (AUC) of the receiver operating characteristic (ROC; Manel et al., 2001; Brotons et al., 2004; Thuiller et al., 2005; Peterson et al., 2008) was obtained in IBM SPSS Statistics software (v. 22, International Business Machines Corp., New York, USA), using the obtained

Measure	**Formula**
Overall accuracy	$\frac{TP+TN}{n}$
Sensitivity	$\frac{TP}{TP+FN}$
Specificity	$\frac{TN}{TN+FP}$
Kappa statistic	$\frac{\left(\frac{TP+TN}{n}\right)-\frac{(TP+FP)(TP+FN)+(FN+TN)(TN+FP)}{n^2}}{1-\frac{(TP+FP)(TP+FN)+(FN+TN)(TN+FP)}{n^2}}$
TSS	$Sensitivity + Specificity - 1$
F_{pb}	$\frac{2\times TP}{TP+FN+FP}$

Table 2. Parameters used in the evaluation of individual models and the weighted average consensus model. Abbreviations: TSS, True Skill Statistic; WA, weighted average; n, total number of cases; TN, true negative; FP, false positive; TP, true positive; FN, false negative (*sensu* Li and Guo, 2013).

measures as the probability that one score associated to a random presence site is higher than the probability of a random pseudoabsence site (Elith, 2000). The F_{pb} index, specifically relying on presences and pseudoabsences (Li and Guo, 2013), was then calculated from the contingency tables. In this way, the regions where the pathogen's establishment is possible but did not occur or was not yet detected, were not considered. For each category of model construction, only those that performed the highest F_{pb} measures were used to generate relative suitability surfaces in the study area and a qualitative comparison in Quantum GIS (QGIS Development Team, 2013) was performed. A quantitative comparison was performed on the basis of the percentages of agreement among the predicted probabilities of selected models that were calculated on the whole dataset considering the conventional threshold of 0.5 between presence and pseudoabsence (Liu et al., 2005).

The weighted average consensus model and spatially realistic probability

Considering that the nine models gave partially different probability maps but performed very well in the comparison with the test set, rather than selecting just one as definitive, their prediction outputs were combined using a consensus modelling framework procedure (Araújo et al., 2005; Marmion et al., 2009a; Grenouillet et al., 2011). Furthermore, this approach can enable more robust decision-making in the face of uncertainty, in particular in a conservation planning context (Araújo and New, 2007). Therefore, a weighted average (WA) was calculated on the previous evaluation of the selected modelling techniques but because pseudoabsences cannot be considered as confirmed *H. fraxineus* absences, instead of using conventional AUC values as weights (Marmion et al., 2009a, 2009b), the new measure of F_{pb} was exploited:

$$WA_j = \frac{\sum_{ij}(F_{pb_i} \times P_{ij})}{\sum_i F_{pb_i}}$$

with P representing the predicted relative suitability (*sensu* Phillips and Elith, 2013) of the single model i for each grid cell j. The performance of the new WA consensus model was assessed with the same statistics and test set used for the individual models described above.

Because the natural spread of a pathogen is an intrinsically spatial process

(Bian, 2004), the spatially explicit model for *H. fraxineus* in the study area was produced to identify possible suitable areas not reachable with a natural spread process, using the following procedure. According to a precautionary approach, the patches resulting as suitable for the pathogen or useful as transitional zones were selected in Quantum GIS (QGIS Development Team, 2013) from the potential map obtained from the ensemble modelling technique (Marmion et al., 2009a, 2009b). This operation was made through a binary transformation and considered the threshold maximising the True Skill Statistic (Barbet-Massin et al., 2012; Jimenez-Valverde and Lobo, 2007). A script in R (R Core Team, 2013) was written *ad hoc* to generate a network (Wassermann and Faust, 1994; Brooks et al., 2008; Firestone et al., 2012) among neighbour polygons with a distance lower than 1.3° (approximately 120 km) and with a safety factor of one (two times the maximum spread distance indicated in the literature, given that the natural spread rate of *H. fraxineus* can reach 60 km/year (Timmermann et al., 2011; Solheim et al., 2011). R code for the construction of the spatially explicit model is included as part of the ANNEX 3. The spread of the pathogen from the presence points in the network was then simulated in R (100 iterations over time). In this way, the final prediction excluded the areas potentially prone to natural spread in the WA consensus model, but not the regions gradually reachable from presence areas.

Relative importance of predictor variables

The contributions of each environmental variable to the construction of the models used in the WA consensus procedure obtained from the software were merged in a single relative importance value (OI, Overall Importance) to achieve a more readable result. This arrangement was achieved by computing a weighted average (Microsoft Excel v. 2007, Microsoft Corporation, Redmond, USA; Microsoft, 2007) using the F_{pb} value associated with each individual model, similarly to the construction of the WA consensus model:

$$Overall\ Importance\ of\ Predictor_j = \frac{\sum_{i,j}(F_{pb_i} \times Importance\ of\ Predictor_{i,j})}{\sum_i F_{pb_i}}$$

where j represents the predictor and i the individual model.

Results

Extension of the study area

The shapefile corresponding to the study area included most of Europe plus neighbouring countries, from a northern limit in southern Scandinavia to some parts of north-western Africa and Anatolia, from Ireland and Portugal to western Russia and northern Iran (Figure 5).

Chosen predictors

The collinearity test based on the Pearson correlation allowed the number of predictors to be reduced from 122 to 50, as reported in Table 1. In particular, the maximum and minimum temperature at a height of 2 m, snow cover and runoff were discarded from further analyses. From the intersection of the maps with the grid, 4576 background samples were obtained and then reduced to 531 to allow model building.

Model fits and comparison

During model building, each type of algorithm was optimised and the best final set parameters are reported as part of the ANNEX 4. The relative efficacy of the models on the test set was evaluated by comparing contingency tables (Figure 6, see ANNEX 5 for a deepen explanation) and a series of parameters (Table 3). Among the models, SVM and MLP built with boosting or bagging procedures and Maxent achieved the highest measures of overall accuracy, sensitivity, Kappa statistic and TSS. In the comparison of the algorithms on the basis of the ROC curve (Figure 7), the SVM and the two MLP models were the best performing in predictive accuracy. This result was confirmed by the respective AUC values (AUC>0.9; Table 3). Considering specificity and F_{pb}, the values covered a greater range, indicating that LOG with first order interactions and CHAID bagging models performed significantly worse than the others in the same categories. As a result, the models selected for the WA consensus model for each category of construction on the basis of F_{pb} measures were GLM, SVM, CHAID built with boosting procedure, Artificial Neural Network MLP with boosting procedure, and Maxent. The WA consensus model often achieved higher performances on the basis of the evaluation parameters than the single models used for its construction.

The projections of the selected models in the QGIS software were visually

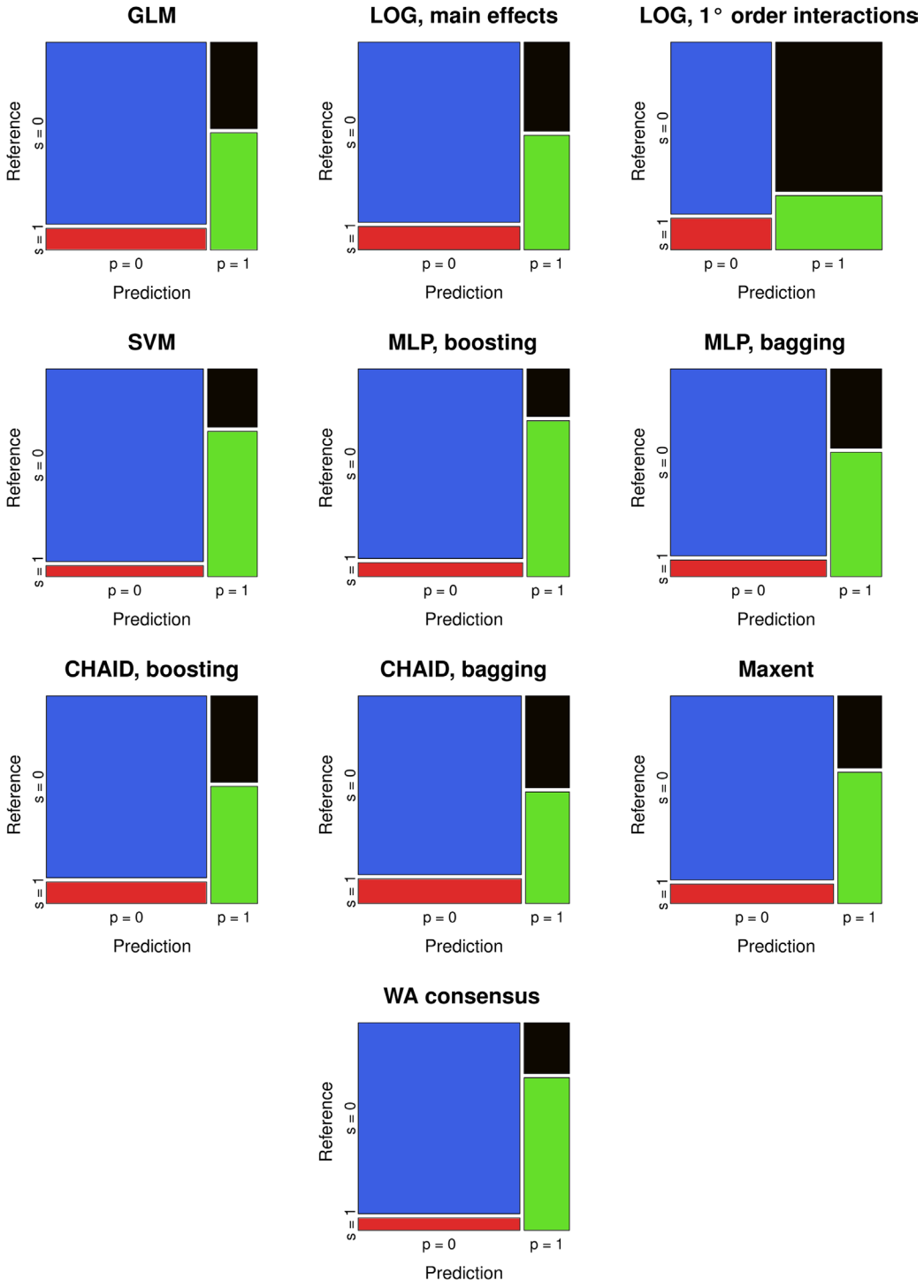

Figure 6. Mosaic plots for every single model and weighted average (WA) consensus model. Mosaic plots were obtained from the contingency tables used to compare predicted probabilities with the test set. In each plot, "s = 0" and "s = 1" stand for "pseudoabsence" and site with symptomatic ashes in the reference set (test set); "p = 0" and "p = 1" indicate predicted the unsuitability and suitability scenario by the single model. The size of the box obtained from the combination of every "p" with "s" is proportional to the number of cases for each contingency table case. In particular,

the blue, black, green and red boxes indicate the proportion of "true negative", "false positive", "true positive" and "false negative", respectively. Abbreviations: GLM, Generalised Linear Model; LOG, Logistic Regression Model; SVM, Support Vector Machine Model; MLP, Multilayer Perceptron Artificial Neural Network; CHAID, Chi-squared Automatic Interaction Detector Model; WA, weighted average.

Model	**Overall accuracy**	**Specificity**	**Sensitivity**	**Kappa statistic**	**TSS**	**AUC**	**F_{pb}**
GLM	0.81	0.83	*0.71*	0.49	0.54	0.87	**0.88**
LOG, main effects	0.81	0.88	0.58	0.45	0.46	0.88	0.80
LOG, 1° order interactions	0.55	0.52	0.65	0.11	0.17	0.56	0.47
SVM	*0.89*	*0.91*	*0.81*	*0.69*	*0.72*	*0.92*	***1.22***
MLP, boosting	*0.90*	*0.94*	*0.74*	*0.69*	*0.68*	*0.92*	***1.21***
MLP, bagging	0.84	0.88	*0.71*	*0.56*	*0.59*	*0.92*	*0.98*
CHAID, boosting	0.81	0.88	0.67	0.49	0.54	0.85	**0.88**
CHAID, bagging	0.81	0.88	0.55	0.43	0.43	0.83	0.76
Maxent	*0.85*	*0.90*	0.65	0.55	0.55	0.90	**0.95**
WA consensus model	*0.90*	*0.93*	*0.77*	*0.70*	*0.70*	*0.94*	***1.23***

Table 3. Performances of the individual models and the weighted average consensus model. Performances were computed on the test set considering overall accuracy, specificity, sensitivity, Kappa statistic, True Skill Statistic (TSS), the area under the curve (AUC) of the Receiver Operating Characteristic (ROC) and F_{pb} (Table 2). The best four values for each parameter are italicised; the bold values indicate the best model for each category (according to those presented in the Materials and Methods section) on the basis of F_{pb} measures. Abbreviations: GLM, Generalised Linear Model; LOG, Logistic Regression Model; SVM, Support Vector Machine Model; MLP, Multilayer Perceptron Artificial Neural Network; CHAID, Chi-squared Automatic Interaction Detector Classification Tree; WA, weighted average.

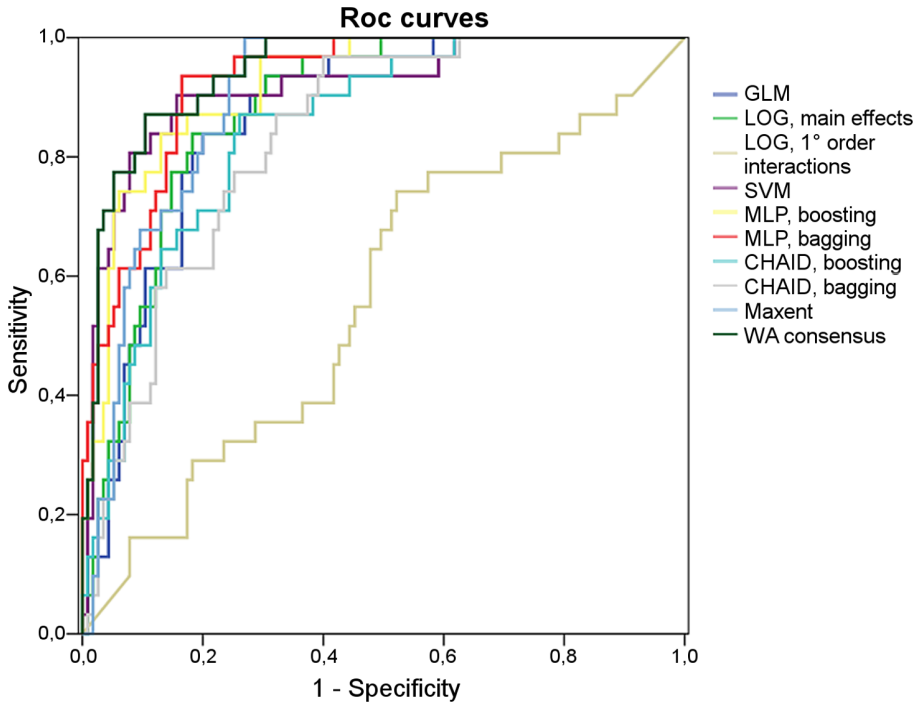

Figure 7. ROC curves for the individual models and for the WA consensus model. Sensitivity is plotted against the corresponding proportion of false positives (1-specificity), at various threshold settings. Abbreviations: GLM, Generalised Linear Model; LOG, Logistic Regression Model; SVM, Support Vector Machine Model; MLP, Multilayer Perceptron Artificial Neural Network; CHAID, Chi-squared Automatic Interaction Detector Classification Tree; WA, weighted average.

different both in predicted extent and in the levels of the relative probabilities (Figure 8). In particular, the GLM forecast a wider potential area, with eastern extremes in the Moscow region (Figure 8, A). In the SVM, the potential area was more restricted and had higher associated relative probabilities; Figure 8, B). A similar result was obtained for the MLP, but with more irregular and fragmented areas (Figure 8, C) in addition to the CHAID regression tree model (Figure 8, D). The spatial pattern associated with the Maxent model was completely different and consisted of a smoother and larger potential distribution with a low relative suitability of presence, which also reached some southern zones in the study area (Figure 8, E).

Although the models tended to differ in the magnitude of predicted relative probabilities, agreement was reached by all the models in highlighting the central, northern and eastern Alps, Baltic States, southern Finland and the zone including Slovakia and southern Poland as more suitable areas for the pathogen as potential scenarios.

Considering the quantitative comparison among these models on the basis of predicted relative probabilities in the whole dataset after applying the 0.5 threshold, the percentage of agreement varied from 86.3 % to 93.4 %, whereas the accordance of the models with the WA consensus model achieved higher percentages (89.4 % - 96.7 %; Table 4).

Model	GLM	SVM	MLP, boosting	CHAID, boosting	Maxent	WA consensus model
GLM	-	88.4	88.8	86.3	87.9	89.4
SVM	88.4	-	93.4	91.7	89.9	96.3
MLP, boosting	88.8	93.4	-	91.6	90.0	96.7
CHAID, boosting	86.3	91.7	91.6	-	89.1	94.4
Maxent	87.9	89.9	90.0	89.1	-	91.5
WA consensus model	89.4	96.3	96.7	94.4	91.5	-

Table 4. Percentages of agreement in relative probabilities predicted by selected individual models and the weighted average consensus model on the whole dataset. The table reports the agreement among relative probabilities predicted by the best models for each category chosen according to the F_{pb} measures reported in Table 3. Predicted relative probabilities are rounded to 0 (pseudoabsence) or 1 (presence) using the conventional threshold of 0.5 before percentages' computation and comparison. Abbreviations: GLM, Generalised Linear Model; SVM, Support Vector Machine Model; MLP, Multilayer Perceptron Artificial Neural Network; CHAID, Chi-squared Automatic Interaction Detector Classification Tree; WA, weighted average.

WA consensus and spatially realistic models

The potential map of *H. fraxineus* drawn from the WA consensus model appeared to be an intermediate result in comparison with previous models (Figure 9, A): the areas at major risk of spread ($p>0.7$), such as the central and eastern Alps, Austria, Switzerland, eastern France, central Ukraine, southern and northern Poland, northeastern Germany, southern Sweden and Finland, central Denmark, southeastern England and the Baltic States, were confirmed and connected by areas of low-medium relative probabilities ($0.25<p<0.7$).

The final map obtained from the network analysis (threshold obtained for TSS

maximisation 0.25) represented the spatially explicit distribution for *H. fraxineus* (Figure 9, B), with the predicted distribution overlapping the greater part of the WA consensus map but with some patches, such as in the Pyrenees and Caucasus, which were not considered, being not gradually reachable from the potential area in Central Europe.

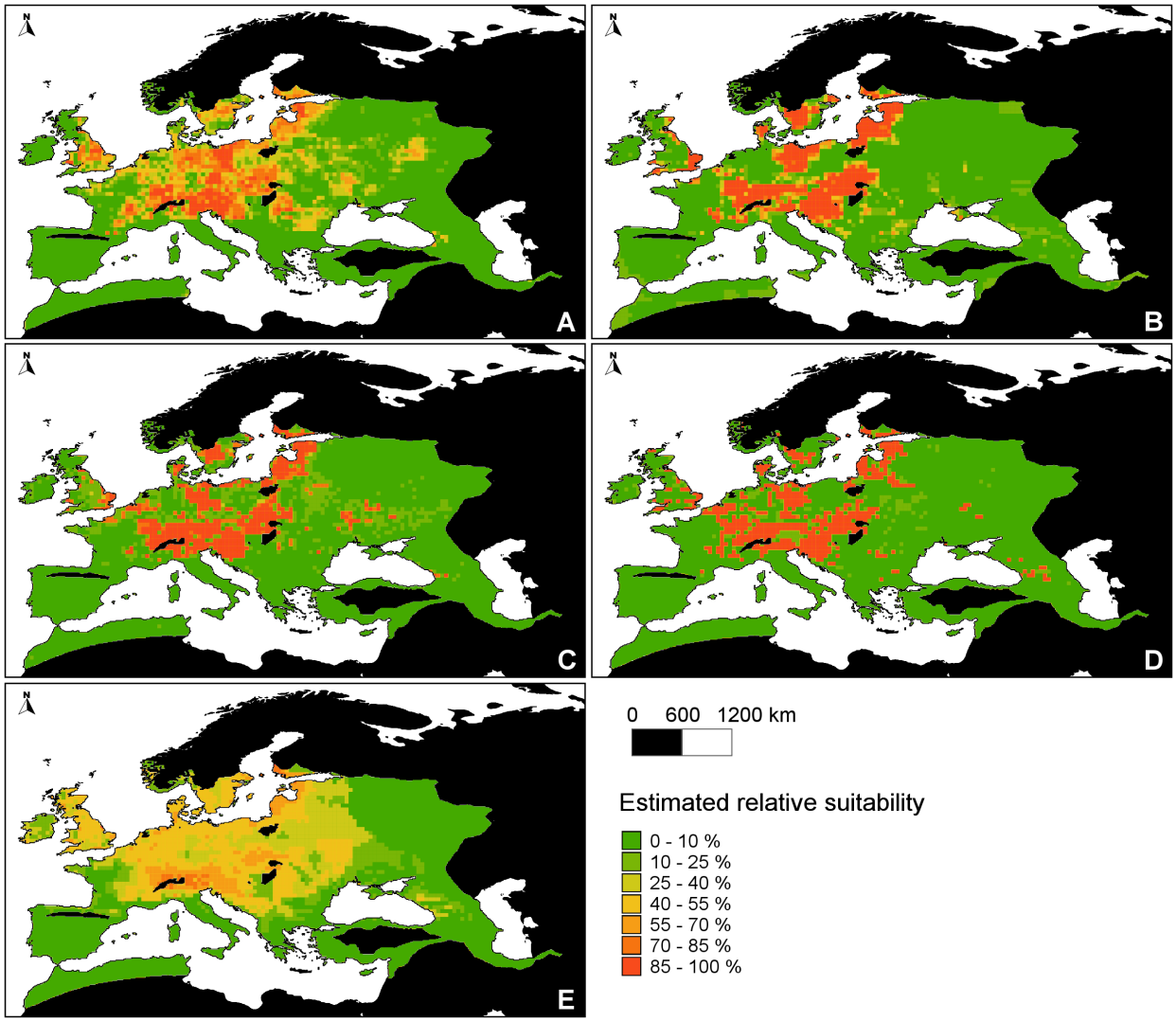

Figure 8. Estimated spatial distribution of *H. fraxineus* in Europe according to the individual models. According to the legend, different colours represent different levels of predicted relative suitability. A, Generalised Linear Model (GLM). B, Support Vector Machine (SVM). C, Artificial Neural Networks Multilayer Perceptron (MLP), with boosting building. D, Chi-squared Automatic Interaction Detector (CHAID) Classification Tree, with boosting procedure. E, Maxent.

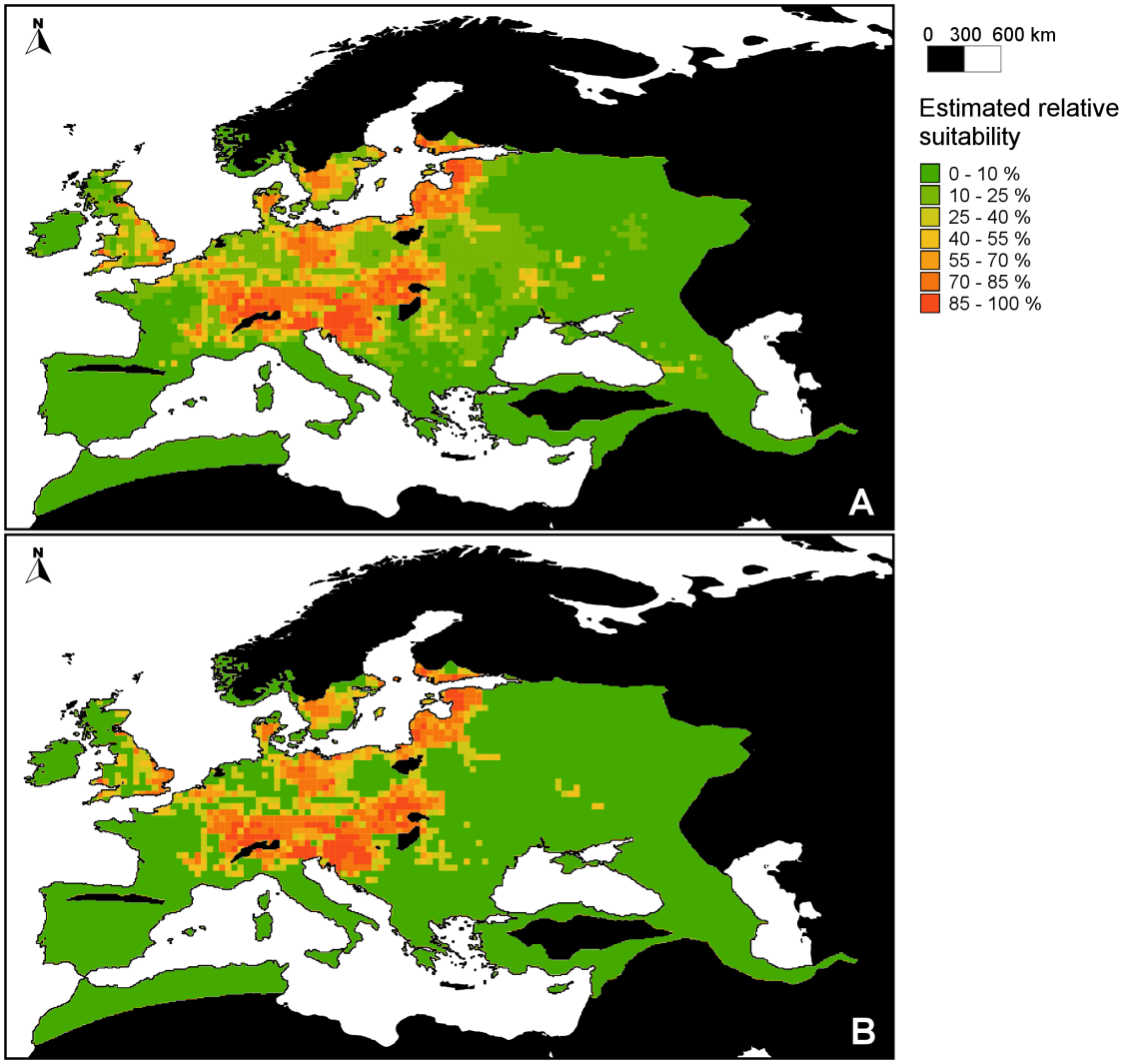

Figure 9. Estimated spatial distribution of *H. fraxineus* in Europe according to the final models. According to the legend, different colours represent different levels of predicted relative suitability. A, WA consensus model. Ensemble values were calculated using a weighted summation approach where predictions from individual models were combined on the basis of individual model valuation. B, Spatially explicit model, obtained from the network analysis.

Environmental variables associated with the natural spread of *H. fraxineus*

Important variables in creating model fits were consistent in all models except for Maxent. Of the 50 predictors, the most important ones (OI $\geq$ 2, OI = Overall Importance) are reported in Table 5. Precipitation in July and August were the two most important variables, with OI values of 7.2 and 12.1, respectively. Precipitation in June and soil water content in August were also relatively strong

predictors correlated with the occurrence of ash dieback (OI = 5.7 and 5.6), while temperature in July and August played a moderate role (OI = 3.9 and 3.2). In general, apart from elevation (OI = 2), the other predictors represented averages during the summer months. In particular, when compared to the average values of the whole study area, the presence of *H. fraxineus* was associated with a low mean temperature between June and September (16.6 °C), abundant summer precipitation (> 80 mm) and higher soil moisture content (> 30 %).

Environmental predictor	**Overall importance**	**Mean value in presence dataset**
Elevation	2.0	359.2 m
Mean temperature, January	2.3	-0.5 °C
Mean temperature, June	2.3	16.8 °C
Mean temperature, July	*3.9*	18.1 °C
Mean temperature, August	*3.2*	17.9 °C
Mean temperature, September	2.8	13.7 °C
Frost day frequency December	2.6	21.7 °C
GDD November-March	2.0	1743.1 °C
Precipitation, May	2.5	77.8 mm
Precipitation, June	*5.7*	91.4 mm
Precipitation, July	**7.2**	100.4 mm
Precipitation August	**12.1**	93.8 mm
Precipitation, September	2.0	82.1 mm
Soil moisture, March	2.2	0.35
Soil moisture, July	*3.3*	0.31
Soil moisture, August	*5.6*	0.30

Table 5. Overall Importance (OI) of environmental predictors included in model building. Variables with OI>2 are shown; predictors with OI>6 are in bold, italicised text indicates the variables with 3<OI<6. The average value for each predictor

considering the dataset of *H. fraxineus* presences are displayed in the right hand column. Abbreviation: GDD, Growing Degree Days.

Discussion

Due to the rapid spread of *H. fraxineus* in Europe reported in recent years (EPPO, 2014; Gross et al., 2014a), this study was performed to provide a spatial prediction of the vulnerability of indigenous ash tree species in Europe, considering the distribution of hosts and the main environmental factors associated with naturally infected sites.

Among the nine algorithms compared, the Support Vector Machine (SVM), Artificial Neural Network Multilayer Perceptron (MLP) with boosting procedure and Maxent models achieved the highest measures of specificity, Kappa statistic, Area Under the Curve (AUC) and F_{pb}, demonstrating that they fit the test set better than the other models, which allows projections of observed patterns into independent situations and minimises over-fitting of data (Araújo and Guisan, 2006). Sensitivity, an essential measure in models with presences and pseudoabsences, was significantly higher in the Generalised Linear Model (GLM), SVM and MLP Artificial Neural Network models. The generally higher performance of machine-learning methods, most likely due to peculiar advantages, such as robust parameter estimates, model structure learned from data and easy fitting of complex interactions, in spite of considering the use of pseudoabsences in models evaluation (Chefaoui and Lobo, 2008), was therefore confirmed (Valle et al., 2013).

Among the performance measures considered, prominence was given to the F_{pb} statistic. This accuracy assessment was recently proposed by Li and Guo (2013) for presence-only modelling, to specifically consider presences and pseudoabsences instead of true absences in the confusion matrix (Li and Guo, 2014). Given that the performance of such models with regard to F_{pb} were quite robust, but that their predictive maps partially differed in the extension and magnitude of relative suitability of the pathogen's presence, in accordance with Stohlgren et al. (2010), the consensus ensemble forecast was calculated as the weighted average of the best models, highlighting the areas of agreement among models as expected and thereby minimising the weakness of any given algorithm (Araújo et al., 2005; Araúio and New, 2007; Marmion et al., 2009b). The resulting model, highlighting the areas

suitable for the pathogen, generally outperformed any single algorithm based on the evaluation parameters (mainly Kappa statistic, AUC and F_{pb}) and suggested a potential distribution map with higher risk for the central and eastern Alps, Baltic States, southern Finland and Sweden, Slovakia and southern Poland. This approach can enable more robust decision-making in the face of uncertainty (Araúio and New, 2007), however, as suggested by Elith et al. (2010), caution should be taken in selecting models for an ensemble forecast. An understanding of the data, single models and predictions should not be underestimated, especially in the case of a climate change context.

Species Distribution Models (SDMs) and ensemble forecasting lead to interesting conclusions on the ecological appropriateness of some areas to the potential pathogenic spread of *H. fraxineus* (Peterson, 2003). To take into account the dispersal limitation (Svenning and Skov, 2004) and obtain a more realistic projection, a novel approach to the network analysis was implemented, which considered the potential map obtained from the ensemble modelling technique and the points where the disease presence can be considered as derived from a natural infection. In this way, most of the edging areas of the *F. excelsior* chorological map resulted as unsuitable for a natural spread in the final scenario (i.e., western Ireland, part of France and northern Spain, all the southern areas in central Italy, the Balkans and northern Turkey and western areas in Russia, the Caucasus and Iran). The reported consensus ensemble forecast potential distribution map may therefore indicate the areas where the trade of ash species should be under particular control. In any event, caution should be taken in transferring predicted results from a wider scale to a more local scale (Boychuk et al., 2004; Münzbergová, 2004). The disease in Europe most likely originated from a single introduction event of the pathogen of at least two individuals with compatible mating types and was first observed in the early 1990s in Poland (Gross et al., 2014b), although there is an hypothesis of the introduction of the pathogen together with the importation of *F. mandschurica* to Estonia (Drenkhan et al., 2014). The computed network analysis based on current presence points aimed to deliver realistic predictions, so the potential distribution in the case that the disease originated in others regions was not implemented.

The expected spread to *F. angustifolia* largely resulted as low, except for some areas in northern Italy, Slovenia, Croatia, Bosnia-Herzegovina and Romania,

confirming the hypothesis of Gross et al. (2014a), who reported that the epidemic rate in Europe is slowing down, the sill of a sigmoid function of spatial growth against time has been reached. Similar studies on invasive pest modelling suggest that environmental conditions may serve as a constraint limiting the spread in respect to the hosts' distribution. For instance, Podger et al. (Podger et al., 1990) considered the establishment of *Phytophthora cinnamomi* in potential areas with annual mean temperature < 7.5°C and annual mean rainfall < 600 mm as unlikely. Moreover, Koch and Smith (2008) estimated the potential spread of non-native *Xyleborus glabratus* in the southeastern U.S. and concluded that climatic conditions could prevent the spread from coastal plain to eastern interior forests.

As a result of the modelling, precipitation, soil moisture and air temperature were shown to be significantly more influential than other predictors in model building of the potential distribution of *H. fraxineus*. In particular, the species distribution appeared to be highly dependent on abundant rainfall and high soil moisture content in the summer months, confirming the hypothesis of more intense ash dieback near water courses or in high soil moisture sites (Cech, 2008; Ogris, 2008) and supporting the hypothesis of Gross et al. (2012) about the importance of free water for the fertilisation of the *H. fraxineus* anamorph on petioles. Summer mean temperatures were also relevant for model construction of the pathogen niche, consistent with available information on the species (Hauptman et al., 2013). In an ecological and biological interpretation, the temperature ranges highlighted by the models could be necessary for apothecia production, known to occur from May to October, with a peak in July, with a minimum temperature of 1.1°C and optimum temperature of 22°C (Ogris, 2010; Timmermann et al., 2011). Considering the low January mean temperature in areas where the species was present (-0.5°C), various studies indicate that the fungus can develop within the plants over the winter, causing necrosis (Sansford, 2013), and the mycelium tolerates freezing at -20°C for at least two months and can even survive-70°C for at least one month (Gross et al., 2014a). In addition, conidial sporulation is favoured *in vitro* by temperatures between 5 and 15°C (Kowalski and Bartnik, 2010) and was observed in nature in autumn on ash rachises in the ground litter (Kirisits et al., 2009).

Modelling was directly trained on the entire study area in order to cover all the environmental gradients in the distributions of *F. excelsior* and *F. angustifolia* and to avoid underestimating climatic factors in delimiting species' distribution (Barve

et al., 2011). Moreover, attention must be paid in considering relative suitability predictions of the models, because of the possible lack of equilibrium, which is typical of an invasive pathogen not yet reaching its full potential distribution (Elith et al., 2010). For this reason, further reports of ash dieback over time, including the probable original Asian distribution (Zhao et al., 2012; Zheng and Zhuan, 2013), could be easily taken into account to enlarge the boundaries of the Grinnellian niche (closer to equilibrium, according to Pulliam, 2000; Anderson et al., 2004; Václavík and Meentmeyer, 2012). The availability of a wider time series, including data on ash dieback severity and host abundance, will allow the consideration of the spread dynamics of the disease in the context of different landscapes and in a climate change scenario (Rosenzweig et al., 2001; Anderson et al., 2004; Chakraborty, 2005). More detailed mathematical analyses are in progress, to identify the specific high performance components in the machine learning models able to describe the biological and ecological complex interactions involved in the expression of the disease.

CHAPTER III

Efficacy tests on commercial fungicides against Ash dieback *in vitro* and by trunk injection

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Abstract

Ash dieback, caused by *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya, comb. nov. (basionym *Chalara fraxinea* T. Kowalski, synonym *Hymenoscyphus pseudoalbidus* Queloz et al.), has emerged as a critical disease in urban areas and in the forests of many European countries. This study was conducted to evaluate six fungicides for their potential to control the disease. *In vitro* assays with different concentrations of the products against five different strains of the pathogen, illustrated that thiabendazole, propiconazole and allicin exhibited lower median lethal doses, procloraz completely killed half of the samples at higher concentrations, whereas copper sulphate and potassium phosphite were totally ineffective. Subsequently, the antifungal activities of the best three compounds were investigated *in planta* against *H. fraxineus* by trunk injection. The rate of necroses development following artificial inoculation of 24 *F. excelsior* was significantly slowed down in the growing season by the treatment with thiabendazole and allicin. In the phenological phase and climatic conditions tested, and with the chosen formulation and injection method, propiconazole injections were impracticable. The results of this study, along with some technical suggestions for application in the field, support the idea of using organic and chemical endotherapic products to combat ash dieback symptoms in *Fraxinus* spp., with the safe and very low impact method of trunk injection.

Keywords

allicin, *Chalara fraxinea,* endotherapy, *Fraxinus excelsior*, *Hymenoscyphus fraxineus*, thiabendazole.

Introduction

Over the last 14 years, an increasing decline in ash trees (*Fraxinus excelsior* L. and *F. angustifolia* Vahl) has been noted in Northern and Central Europe. According to Kowalski (2006) and Queloz et al. (2011), this has been caused by the Ascomycete *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya, comb. nov. (basionym *Chalara fraxinea* T. Kowalski, synonym *Hymenoscyphus pseudoalbidus* Queloz et al.). Also pathogenicity was noted against the European *F. ornus* L., the North American *F. nigra* Marsh., *F. pennsylvanica* Marsh., *F. americana* L. and the Asian *F. mandschurica* Rupr. (Drenkhan and Hanso, 2010; Kirisits et al.,

2010).

All age classes are affected, resulting in terminal decline. Infection takes place on leaves or at the leaf rachises, after wind dispersal of ascospores in summer from apothecia developed from pseudosclerotial plates in infected leaf remnants in the litter (Cleary et al., 2013; Gross et al., 2014a). Infected leaves desiccate and the pathogen develops inside the stem, spreading into the phloem below the bark, into the parenchymatic rays and into the xylem, causing a brown discoloration in the wood followed closely by crown dieback (Schumacher et al., 2010; Dal Maso et al., 2012; Gross et al., 2014a). Due to the ease of its spread and pathogenicity, the fungus was included in EPPO Alert List (EPPO, 2007).

During recent years, research has focused on the study of the *in vitro* mycological characteristics (Brasier and Webber, 2013; Kirisits et al., 2013), apothecia and ascospores role in the disease (Gross and Holdenrieder, 2013; Hietala et al., 2013; Kowalski et al., 2013), different host susceptibility, genetic variability of the pathogen (Kraj and Kowalski, 2013; Stener, 2013; MacLean, 2014; McKinney et al., 2014; Thomasset et al., 2014), pathogen detection techniques (EPPO, 2013b; Gherghel et al., 2013; Pham et al., 2013) and, finally, the ecological consequences of the disease (Pautasso et al. 2013; Lõhmus and Runnel, 2014; Lygis et al. 2014).

Attention is now being centered on phytosanitary protection of ash trees from the pathogen. At the present time there are no effective measures to control the disease, but biosecurity protocols on disinfection to prevent the spread of *H. fraxineus* have been recommended. In particular, Cooke et al. (2013) proposed various physical and chemical methods to restrict the production and spread of ascospores, including the removal of plant debris from infected sites, preventing movement of infected plant material to new sites, the use of disinfectants to treat contaminated footwear, clothing and equipment and the use of fungicides and biocides for the treatment of infected debris. In addition, hot water treatments were suggested by Hauptman et al. (2013) for the disinfection of plant propagation material or growing plants, considering the sensitivity of *H. fraxineus* to relative high temperature.

Until now there are no complete reports on fungicides effective against the disease (Cooke et al., 2013), but the potential for a cure was considered to be high with procloraz and carbendazim, being able to stop the production of apothecia after fungicidal treatments (Hauptman et al., 2012).

Considering the lack of information on phytosanitary measures against ash dieback, the first aim of this study was to ascertain the *in vitro* lethal dose of six commercial fungicides against the pathogen. The best performing ones were then used for trunk injections (Tattar et al., 1998; Young, 2002; Takai et al., 2003) in artificially infected trees, in order to determine their potential to control the disease *in planta*.

Materials and Methods

In vitro experiments

Commercial fungicidal formulations of six active ingredients (thiabendazole, procloraz, propiconazole, allicin, potassium phosphite and copper sulphate; Table 6), corresponding to an equal number of chemical classes (Benzimidazole, Imidazole, Triazole, Thiosulfinate, Potassium Phosphonate, Copper compounds), were tested *in vitro* for their effect against five *H. fraxineus* strains (Table 7) previously selected among the ones available in the TeSAF herbarium for their high pathogenicity, according to Ogris et al. (2009).

Each fungicidal agent was diluted with sterile de-mineralized water (100 %, 85 %, 65 %, 50 %, 35 %, 15 %, 5 %, 1 %, 0.1 %, 0.01 %, 0.001 %, 0.0001 %, 0 %), and 0.35 mL not buffered suspension was homogeneously spread on the surface of 10 mL PDA (Potato Dextrose Agar, Difco Laboratories, Detroit, MI, USA) in 94 mm diam. Petri dishes (Taiga et al., 2008), accounting 25 replicates per treatment.

After growing the fungal strains on PDA for three weeks at 20±1 °C in the dark, 8 mm diameter. plugs were removed from the actively growing colony margins, then placed top- down onto five plates per treatment (Aloj et al., 1993; Secor and Rivera, 2012). After an incubation at 20±1 °C in the dark for 3 days, plugs were transferred to unamended PDA in the same conditions (Aharoni et al., 1997; Allen et al., 2004; Suleiman, 2010), and fungal growth was checked weekly using a microscope (up to 200x) for five consecutive weeks.

Growing colonies were classified as “vital”, while those which failed to grow as “dead”. Growth data were statistically elaborated in R cran (R Core Team, 2013) by means of one-way analysis of variance (ANOVA, $p<0.05$) to evaluate the growth between strains at different concentrations. Then, for every strain and product, a regression curve fitting was performed by means of Generalized Linear Model considering a binomial outcome, choosing the best between *logit* and *probit*

Commercial product	Active ingredient	Strength	Manufacturer
TECTO 20S	Thiabendazole	220 g/L	Syngenta Crop Protection S.p.a.
SPORTAK 45 EW	Procloraz	450 g/L	BASF Italia S.p.a
ALAMO	Propiconazole	14.3 %	Syngenta Crop Protection S.p.a.
CONQUER	Allicin	5000 ppm	JCA Limited
FOSFISAN	P_4O_{10} + K_2O	30 %, 20 %	Agrofill by Adriatica S.p.a.
BIOYETHI CU	Copper sulphate	2 %	Summerfruit S.r.l.

Table 6. Commercial products and respective active ingredients tested for their fungicidal effect against *H. fraxineus*.

Isolate name	Location	Sample collector
Cf 1005	Cornuda (TV)	Dal Maso E.
Cf 1032	Fusine (UD)	Ogris N.
Cf 1054	Cessalto (TV)	Frigimelica G.
Cf 1056	Falcade (BL)	Frigimelica G.
Cf 1058	Cencenighe Agordino (BL)	Frigimelica G.

Table 7. Isolates of *H. fraxineus* chosen for *in vitro* experiment.

model on the base of Akaike's information criterion (AIC), the coefficient of determination (R^2) and residual analysis (Secor and Rivera, 2012).

LD50s (lethal dose for 50 % of the colonies; Aloj et al., 1993) were calculated (ANNEX 6) and then compared among the active agents effective for all the 5 strains by means of Multiple Comparison (TukeyHSD, $p<0.05$). Shapiro-Wilk Normality Test and Levene test for homogeneity of variance across groups ($p<0.01$) were performed to check for test assumptions. The three active ingredients that achieved the smallest LD50s were selected for *in planta* trials.

In planta experiments

The experiment was carried out in a forest of Common ash (*F.* excelsior; N 45°50'26", E 11°58'20", 180-230 m asl, Cornuda, TV), where *H. fraxineus* has been present since 2010. Specimens ranged from young to mature trees naturally regenerated, to mature trees planted in the 1970's. Due to the need to infect asymptomatic trees with the pathogen, and restrictions made by the forest owner,

after careful selection 24 ash trees were chosen for the experiment, ranging from 16.7 to 37.8 cm (ave. 26.45 cm) diam. at breast height (dbh).

Artificial inoculations were performed using the indigenous strain *Cf* 1005, previously grown on PDA added with streptomycin (0.5 % w:v) for 60 days at 20±1 °C in the dark. In May 2012, every trunk was wounded 150 cm above the collar with a sterile 7 mm diam. cork borer, penetrating approximately 5 mm, and a plug of the same diameter removed from the colony edge was placed top side inward into the hole, then protected with the bark previously removed.

In June 2013, the edges of the infected wounds were carefully debarked, photographed with a scale bar and their areas were measured by means of ImageJ software (v. 1.46r, Wajne Rasband, National Institutes of Health, USA; Abràmoff et al., 2004). According to both tree diameter and the necrotic areas, the 24 trees were then organized into four comparable groups (Peterson et al., 2009), to be injected with commercial formulations of thiabendazole 24 %, propiconazole 24 %, allicin 80 % (Table 6) and water, as a control.

For the injections, a handheld tool recently developed by the University of Padova (BITE; Montecchio, 2013) was chosen. Preliminary trials to increase the injection speed were made on neighboring trees (*F. excelsior*) in April-May 2013 at different times of the day and with different points of injection (root collar or into the trunk 1.5 m from the soil). Furthermore, as thiabendazole tested formulation is not registered for endotherapy (Table 6), its injection performance at different concentrations of the active ingredient was tested adding a series of chemical adjuvants (acetic acid, acetone, ammonium nitrate, hydrochloric acid, nitric acid, potassium hydroxide) at increasing concentrations, with 10 replicates for treatment (Zwieniecki et al., 2001). Obtained results were applied for the official trial.

The trees selected for the final test were then injected at 100 cm from the ground in 3 equidistant points starting from the opposite side of the inoculation point, with 2 ml/cm dbh of each of the fungicidal agents (ave. ca. 60 ml per tree), with the addition of 1.2 % acetic acid. When uptake was too low, additional injection ports opposite to the infection, and located between the existing ones, were used. The time required for each injection was recorded, too.

The treatments effectiveness was assessed by comparing the dimension of the necrotic area measured on the day of the treatment with those observed after 3, 5 and 7 months (corresponding to the end of the growing season, autumn and winter,

respectively).

To verify the presence and vitality of the fungus, during every survey, four equidistant 3 mm^3 wood samples were collected along the edge of each inoculation point, plated on PDA plus streptomycin (0.5 % w:v) and incubated for 60 days at 20±1 °C in the dark. Isolations were scored as positive when fungal cultures exhibited the typical *H. fraxineus* morphology (Kowalski, 2006).

Statistical analyses focused on the fungal growth rate after treatments. The relative ratios of necrotic areas were computed for every tree for each survey date in comparison with the previous one and processed in R cran (R Core Team, 2013), first performing Shapiro-Wilk Normality Test and Levene test for homogeneity of variance across groups ($p<0.01$), then with Multiple Comparison (TukeyHSD, $p<0.05$).

Results

In vitro experiments

Analysis of variance showed no significant differences among the strains treated with different concentrations of thiabendazole (Tecto 20S; $p=0.912$), copper sulphate (Bioyethi Cu; $p=1$), propiconazole (Alamo; $p=0.98$) and procloraz (Sportak 45 EW; $p=0.281$).

Significant differences among strains were observed for potassium phosphite (Fosfisan; $p=0.0258$) and allicin (Conquer; $p=0.019$), with potassium phosphite at high concentrations inducing a fungistatic effect versus *Cf* 1005 and *Cf* 1032 during the first week, then settling into more uniform pattern of results.

LD50s of the tested products are reported in Figure 10. All strains demonstrated sensitivity to the three synthetic fungicides, procloraz, thiabendazole and propiconazole. Their equations in the logistic model showed a high suitability as confirmed by p and R^2 values. At high concentrations, 1 to 5 g/L, no differences were observed amongst them, but at lower doses thiabendazole was the most effective agent, with calculated LD50 value varying between 0.84 (for more susceptible strains) to 40.7 mg/L (for more resistant ones). *H. fraxineus* was significantly less susceptible to propiconazole (TukeyHSD, $p=0.019$) and to procloraz (TukeyHSD, $p<0.001$), whose LD50 respectively ranged from 28.9 to 158 mg/L and from 0.15 to 0.78 g/L among strains. In addition, fungicidal effects of propiconazole and procloraz significantly differed (TukeyHSD, $p<0.001$).

The organic pesticide allicin was effective against three isolates, in particular, *Cf* 1005, *Cf* 1056 and *Cf* 1058, which were associated to LD50 0.13, 0.11 and 0.09 g/L, respectively.

Both potassium phosphite and copper sulphate were ineffective against the fungus at the concentrations used and consequently it was not possible to fit the regression dose–response model for these agents. Therefore, the LD50 values could not be calculated.

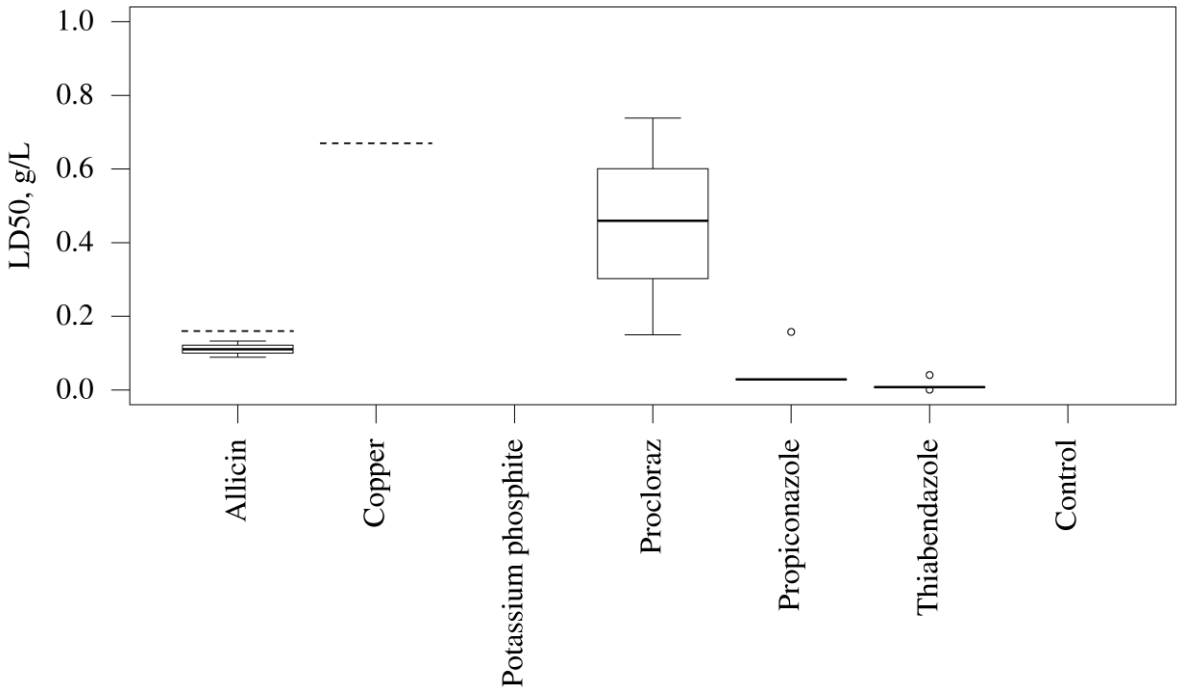

Figure 10. LD50 values calculated for each active ingredient. Boxplots illustrate the variation among *H. fraxineus* strains. Dotted lines indicate the maximum concentration (m.c.) tested for the corresponding active ingredient; when lacking m.c. is higher than 1 g/L.

In planta experiments

In the subsequent paragraph the most relevant results of the preliminary studies on enhancing solution trunk injection in *F. excelsior* are explained.

Considering the point of the treatment on the tree, the injection of water or 26.4 g/L thiabendazole solution was null when performed at the trunk collar and was significantly better (ave 6.3 mL/3 minutes for water, 5.9 mL/3 minutes for thiabendazole, applying a pressure of ave. 100000 Pa) at 1.5 m height. The time of the injection was also fundamental: as indicated in Figure 11, the best

performances for the injection were achieved in early morning (8 00 - 11 00) and then in late afternoon (18 00 - 20 00), whereas the worst results were obtained in the middle of the day and at night. As observed in the last series of preliminary trials, different adjuvants lead to different injection performances. Figure 12 shows most significant results: the addition of 1.2 % acetic acid almost doubled the average speed of the injection of water or thiabendazole solution; hydrochloric acid solution enhanced the performance but with minor efficacy. Acetone and ammonium nitrate additions slowed down the velocity of injection, whereas nitric acid and potassium hydroxide inclusion completely blocked the infusion.

At the time of injection in the forest, all inoculation points showed the presence of visibly developed cankers. These showed great variance in shape and size independent of tree diameter (ANNEX 7).

The length of injection time for each injection point were quite different for each of the selected agents. Acidified water required up to 50 minutes, thiabendazole up

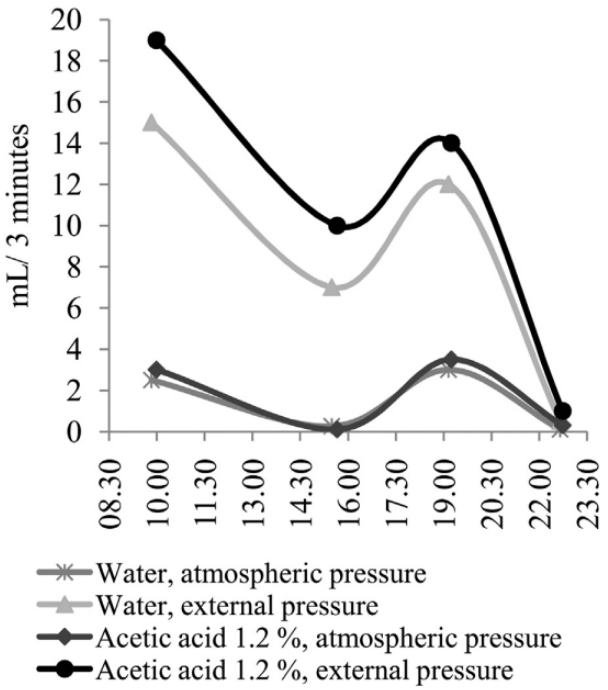

Figure 11. Different performances in injection speed at different moment in the day into *F. excelsior* trunk at breast height (May 2013). Two solutions are tested (water and acetic acid 1.2 %), at atmospheric pressure and at the pressure of the thumb on the plunger (ave. 100000 Pa).

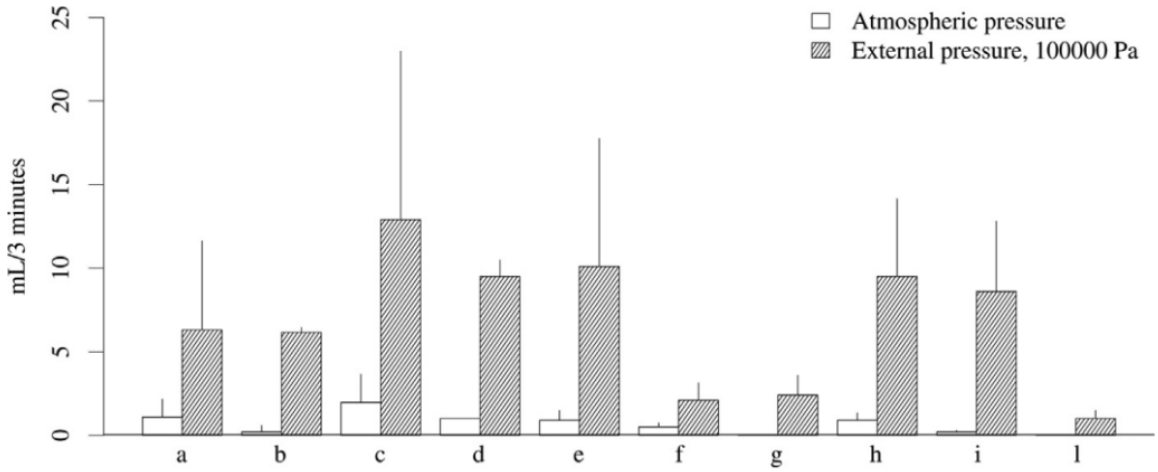

Figure 12. Average quantity of different solutions injected in three minutes at 150 cm from the soil. Milliliters infused at atmospheric pressure (grey columns) and injected at ave. 100000 Pa (in black columns). Error bars: 95 % confidence interval (p-value 0.05, n=10). a = water, b= thiabendazole 26.4 g/L, c= acetic acid 1.2 %, d= thiabendazole 26.4 g/L plus acetic acid 0.4 %, e = thiabendazole 26.4 g/L plus acetic acid 1.2 %, f= thiabendazole 26.4 g/L plus acetone 2 %, g= thiabendazole 26.4 g/L plus acetone 10 %, h = thiabendazole 26.4 g/L plus hydrochloridric acid 0.3 %, i = thiabendazole 26.4 g/L plus hydrochloridric acid 1.5 %, l = ammonium nitrate 4 %.

to 1 hour, allicin up to 6 hours, while propiconazole was never taken up and therefore, given the goal of the research, was discontinued from further evaluations.

Three months after treatment, the necrotic areas were significantly wider than during injections, and none of the products completely blocked the growth of *H. fraxineus*, that was easily reisolated form the necroses' edges after every survey. Nevertheless, when compared with the effect of water injection, thiabendazole and allicin slowed down the necrosis development. In particular, allicin reduced the growth of the necrosis by 55.8 % in average, and thiabendazole by 67.2 % (Figure 13). These results were strengthened by Multiple Comparison analysis (Shapiro-Wilk Normality Test p=0.468; Levene test p=0.069; Table 8), showing that the severity of symptom was significantly lower in both the thiabendazole and the allicin treatments when compared with water-treated trees ($p<0.05$) and that there were no significant differences between thiabendazole and allicin ($p>0.05$).

Five and seven months after the treatments, necrotic areas were no larger than in the first survey and showed an identical trend for the three treatments. Therefore in the statistical analyses, no differences were found significant between the second and the third and between the third and the fourth surveys.

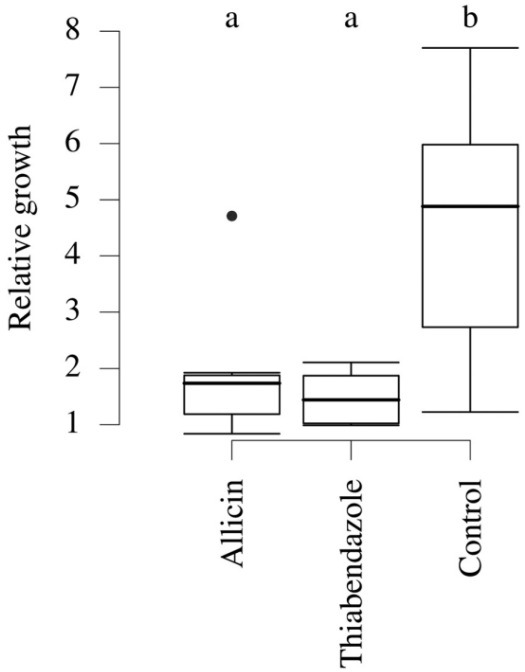

Figure 13. Differences in the relative increase of the necrotic areas 3 months after treatments.

Treatments comparison		Estimated difference	Standard error	p value
Allicin	- Control	2.5162597	0.9566	0.047 *
Thiabendazole	- Control	-3.0289132	0.9566	0.016 *
Thiabendazole	- Allicin	-0.5126536	0.9566	0.855

Table 8. Multiple comparisons (Tukey HSD) between the relative growth of the fungus in the wood from June to September (growing season 2013). Asterisks indicate significant differences ($p<0.05$). Comparisons between September and November, and between November and January surveys were not significant.

Discussion

The research was undertaken to verify if trunk injected commercial fungicides can show a positive effect against ash dieback and significantly reduce the symptoms.

Among the 6 fungicidal agents tested *in vitro* against 5 different fungal strains at 12 different concentrations, the ones containing thiabendazole (Benzimidazole class), propiconazole (Triazole class) and allicin (Thiosulfinate class) proved to be the best performing agents, with the lowest LD50 values. In particular, thiabendazole and propiconazole showed good performances at low concentrations

and a uniform response among strains, whereas allicin showed some differences in sensitivity among isolates, probably due to its organic origin. This is in accordance with previous studies on garlic extracts (e.g. against *Cryptococcus neoformans* and *Mycobacterium tuberculosis*; Fromtling and Bulmer, 1978; Hannan et al., 2011).

In comparison with the precedent fungicides, procloraz (Imidazole class) was only active at higher concentrations. Contrary to expectations, the effectiveness of the commercial formulations of copper, widely used for its fungitoxicity (Gessler et al., 2011), and potassium phosphite, known to be effective against many Oomycetes and some Ascomycetes (Thao and Yamakawa, 2009; Hofgaard et al., 2010), was not demonstrated.

Therefore, according only to the fungicidal effects against the parasite *in vitro* and not to possible host resistance induction (Bécot et al., 2000; Jackson et al., 2000; Machinandiarena et al., 2012; Gozzo and Faoro, 2013), thiabendazole, propiconazole and allicin, characterized by the lowest LD50s, were selected for the following trial *in planta* on 24 previously artificially infected ash trees.

Taking into account current legislation on the use of fungicides (i.e. Directive 2009/128/EC at European level; European Commission, 2009), environmentally safer alternatives to the traditional approach of spraying chemicals should be considered. An increasingly interest in the biological control of fungal diseases is addressed (Santamaría et al., 2007). With this in mind, endotherapic treatments, delivering agents directly into trees, are being more commonly adopted as people become more concerned about the effects of pesticides on humans and the environment generally (Pavela and Bárnet, 2005; Ferracini and Alma, 2008; Tanis et al., 2012). Due to these concerns and to the disease's biology, the selected agents were used for endoxylematic injections, performed by means of a tool, whose functionality is strictly associated to the physiological state of the tree (Montecchio, 2013). As available information on successful trunk injection on *Fraxinus* spp. refers to different application methods, species and climatic conditions (Doccola et al., 2011; Smitley et al., 2010), preliminary trials were performed to assess the best injection procedures considering the tool characteristics, the ash species considered and the local environmental features. Results showed that in order to achieve higher injection speed, treatments should be performed at breast height in early morning or late afternoon; in fact, the lower velocities obtained in the central hours of the day could be explained with stomatal

closure induced by high vapor deficit, with weather conditions of April-May in Northern-Eastern Italy (Iio et al., 2004). Considering the chemicals compounds tested as adjuvants, although great variations were observed depending on weather situation, the addition of a small amount (1.2 %) of acetic acid significantly improved injection velocity, in accordance with Zwieniecki et al. (2001). Furthermore, the presence of acetic acid significantly opposes the adverse influence of heat that could decrease the fungicidal effect for *Allium* plants extract (Yin and Tsao, 1999).

Treatments with propiconazole, although the specific formulation for tree injections used (EPA, 2006, 2011), were impractical in the experimental conditions. This problem could be due to the injection method used, the physiological and health status of the trees, the physiochemical characteristics of the formulation or its dilution, perhaps not fully compatible with the sap dynamics or vessels anatomy of *Fraxinus excelsior* (Choat et al, 2006; Jansen et al., 2009). Unfortunately, the injection process impaired the 6 trees, preventing the substitution with the next best product, procloraz. Allicin injections required up to 7 injection points and on average 5 times the period necessary for a thiabendazole infusion.

Three months after injection, neither thiabendazole nor allicin completely arrested the disease's development over the growing season. This expected result, as seen in many other tree diseases (Perry et al., 1991; Downer et al., 2009; Ivic, 2010; Koch et al., 2010), could be due to product concentration, formulation and distribution inside trees (Tanis et al., 2012; Aćimović et al. 2014). A further explanation could be directly associated to the disease's characteristics. In fact, it was recently reported that *H. fraxineus* produces intrahyphal hyphae inside the wood (Dal Maso et al., 2012), and they could act as barriers impervious to antifungal compounds when present in low concentrations, enabling the fungus to survive (Kim et al., 2004).

Nonetheless, at the dose used, both products significantly slowed down the disease's spread soon after treatment. In fact, unlike the control trees injected with water, 3 months after treatment with thiabendazole and allicin, the growth of the cankers significantly decreased, maintaining the same extensions in the following months until the end of the trial.

Despite the preliminary nature of the described research, this study demonstrated that through the trunk injection of both synthetic and organic

pesticides, the development of ash dieback symptoms can be significantly slowed down for at least few months, including the growing and the dormancy seasons. Further surveys are in progress in order to assess the efficacy in time of one treatment and to determine if these treatments are effective both curatively in naturally infected trees and, preventatively, in healthy ashes exposed to natural inoculum. In addition, the investigations could be extended to a greater number of trees, age classes and genotypes, also comparing different injection methods (McKinney et al., 2012; Stener, 2013; Aćimović et al., 2014; McKinney et al., 2014).

CHAPTER IV

Large-scale fuzzy rule-based prediction of for suitable chestnut ink disease sites: a case study in northeast Italy

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Accepted for publication by Forest Pathology on 17th January 2015 (ANNEX 8): DAL MASO E., MONTECCHIO L., 2014. Large-scale fuzzy rule-based prediction of for suitable chestnut ink disease sites: a case study in northeast Italy. Forest Pathology, I.F. 1.485.

Abstract

In the past few decades, economic interest in the cultivation of chestnuts for both timber and nut production has resurfaced in the Mediterranean area. However, chestnut cultivation has suffered in recent years from the spread of exotic pests, such as the gall wasp *Dryocosmus kuriphilus*, and from the resurgence of previously present diseases, most likely due to anomalous climate dynamics. This is the case with chestnut ink disease (CID), caused by the soil-borne pathogens *Phytophthora cinnamomi* and *P. cambivora*. Scientific and technical support in monitoring and management, that utilises new forecasting approaches incorporating related environmental variables, is therefore essential. The main aim of this study was to develop a mathematical model assessing the potential for the establishment of chestnut ink disease at a large scale. Towards this goal, fuzzy rule-based theory was applied to the environmental variables associated with host presence, pathogens' ecological niches, and ink disease symptoms expression. The effectiveness of the rule-based modelling outcomes, provided with uncertainty maps to facilitate their correct interpretation, was confirmed by detailed field data collected from a large chestnut-growing area where ink disease has been increasing in recent years. The final model gave consistent predictions for disease presence. For this reason, it represents a flexible and valuable decision support tool to forecast which sites are at risk from CID.

Key words: *Phytophthora cinnamomi*, *Phytophthora cambivora*, *Castanea sativa*, monitoring, fuzzy model, epidemics.

Introduction

Phytophthora cambivora (Petri) Buism. and *P. cinnamomi* Rands (Vannini and Vettraino, 2011; Robin et al., 2012) are the soil-borne pathogens responsible for one of the most destructive diseases of sweet chestnut (*Castanea sativa* Mill), the so-called chestnut ink disease (CID; Petri, 1923; Vannini and Vettraino, 2001; Vettraino et al., 2005; Choupina et al., 2014). The disease is spreading in many European chestnut forests and plantations (Turchetti and Maresi 2008; Vettraino et al. 2008; Beccaro et al. 2009; Costa et al. 2011; Woodward et al. 2011), most likely due to the strong relationship between these soil-borne pathogens and environmental features that are changing rapidly due to global climatic change

(Jung et al. 2013; Santini et al. 2013).

The primary CID symptoms are reduced leaf size, dieback of the distal branches, canopy dieback, cracked areas at the base, root and collar necroses, bark detachment, leaking tannic fluid, and the gradual decline and death of the host (ANNEX 1; Vannini and Vettraino, 2001; Vettraino et al., 2005; Vannini et al., 2010; Prospero et al., 2013). Disease progression can be slow, with chestnuts experiencing chronic dieback, or rapid, with large trees killed in a few growing seasons (Jung et al., 2000; Balci and Halmschlager, 2003; Jung et al., 2005). The loss of chestnuts not only entails economic and cultural damage but also compromises the stability of slopes and ridges, leaving them exposed to erosion from rainwater runoff (Maresi and Turchetti, 2008).

For the above reasons, as for other diseases caused by *Phytophthora* spp., risk assessment is recognised as a useful method to rapidly identify, prioritise and manage impacts of pests (Brown et al., 2005; Sansford et al., 2009; Robinet et al., 2012). For this purpose, bioclimatic models have been widely used to assess and predict the occurrence and distribution of pathogens or diseases (ANNEX 2; Venette and Cohen, 2006; Kelly et al., 2007; Ganley et al., 2009; Klopfenstein et al., 2009; Ireland et al., 2013) and their outcomes linked to forest policy, planning and decision-making (Sturrock et al. 2011). Habitat suitability models (environmental niche models) can be constructed utilising spatial analysis methods, which relate the presence or absence of the target species to a set of environmental variables (Iverson and Prasad, 1998; Kelly et al., 2007; Kamino et al., 2012). In recent years, the array of techniques used for ecological modelling has increased (Guisan and Zimmermann, 2000). Fuzzy logic, widely used in engineering and process control sciences (Sugeno, 1985; Von Altrock, 1995), has begun to be applied in biology end environmental sciences (Ayyub and McCuen, 1987; Equihua, 1990). Indeed, fuzzy set theory offers good predictive capability and reasonable estimates of the unknown model parameters inherent in the variables and functions associated with complex ecosystems (Omlin and Reichert, 1999; Adrianssens et al., 2004; Fukuda, 2009).

In plant pathology, this approach found its first applications in disease intensity prediction (i.e., coffee and soybean rust; Kim et al., 2005; Alves et al., 2011), infection simulation (i.e., grapevine downy mildew; Orlandini et al., 2003) and diagnosis (i.e., oilseed crops; Kolhe et al., 2011), and risk assessment (i.e., European

canker of apple; Kim and Beresford 2012), but it has never been applied to forest environments.

In the present study, fuzzy rule-based theory was used to estimate habitat suitability for CID at a large scale in an Italian region with sweetchestnut forests, which have contributed to the economy of mountainous and hilly areas for many centuries. CID has recently been detected in that area (Scattolin et al., 2012), and an increasing number of new foci are being recorded annually. In response to a request by the regional authorities for a practical, user-friendly CID monitoring and forecasting tool, a fuzzy model (FM) was built to consider the environmental variables associated with the ecological niches of the pathogens and disease development. Furthermore, to evaluate the predictive accuracy of the model, uncertainty maps were computed and surveys conducted. The final product stands as a flexible and valuable decision-support system platform, useful for prioritising both periodic monitoring and integrated pest management strategies.

Materials and methods

Study area and environmental predictors

The study area (660 km^2, 93 - 1538 m a.s.l) is located in northeast Italy (Treviso province, Figure 14). According to official literature, the study area covers all of the sweet chestnut phytoclimatic zones, where the species is present in high forest stands (timber and/or fruit production), coppice (timber, poles and firewood) and ornamental plantings (Del Favero et al., 2000; Regione del Veneto, 2006).

A literature research was conducted to construct an ecological knowledge database that was used to select the main input variables relevant to the habitat requirements of the pathogens and to disease development. The potential for *Phytophthora cambivora* or *P. cinnamomi* to spread and to cause CID, as described in the following paragraphs, depends primarily on temperature, annual rainfall, summer droughts, proximity to streams, slope aspect, soil compaction, reaction (soil pH) and organic matter.

Phytophthora spp. survival and disease development are strongly hampered by cold winters (Marçais et al., 1996; Luque et al., 2002; Bergot et al., 2004; Marçais et al., 2004; Vettraino et al., 2005; Desprez-Loustau et al., 2007). A map showing the minimum temperature for the coldest month of the year was obtained from the BIOCLIM database (BIO6, current conditions; Beaumont et al. 2005; Hijmans et

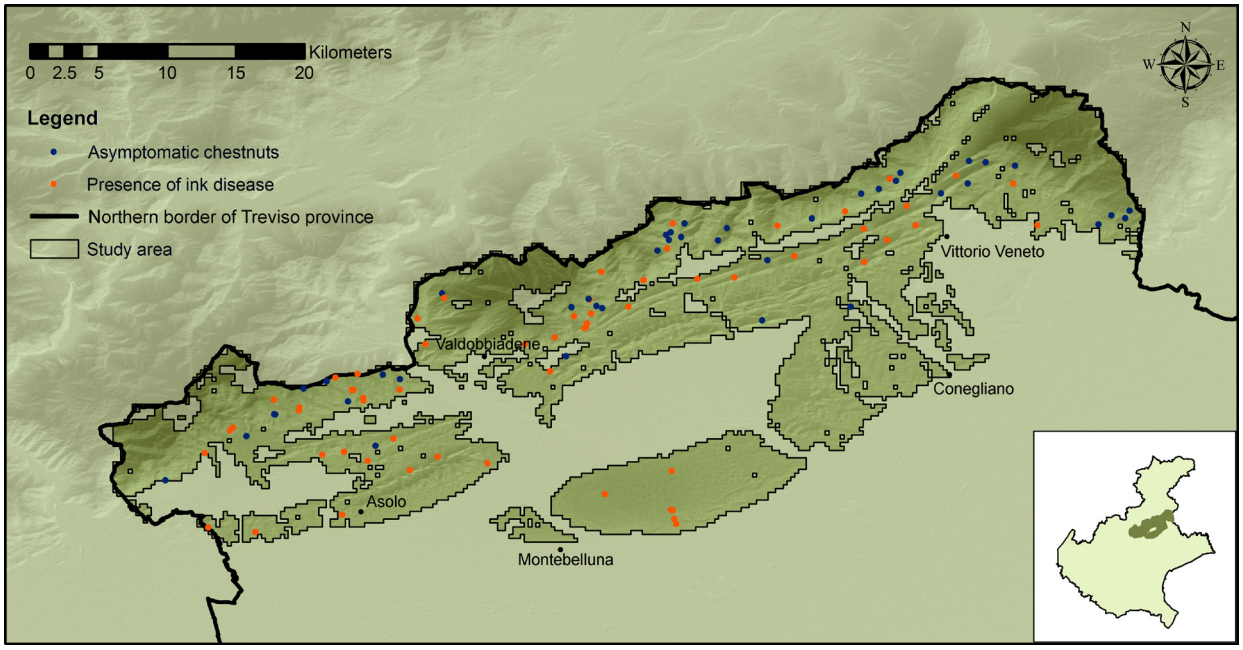

Figure 14. Map of the study area and locations of the 100 points surveyed for chestnut ink disease.

al. 2005; Booth et al. 2014) at 30 seconds resolution (~1 km). Following the procedure reported by Dullinger et al. (2012), the variable was downscaled to a 250 m resolution in Arcmap v. 10.2 (ESRI, 2014) after interpolating the 90 m Shuttle Radar Topography Mission Digital Elevation Model (SRTM DEM) v. 4.0 (Jarvis et al., 2008). The map obtained was then corrected by Kriging on the base of interpolated map of the data acquired from the ARPAV open database (145 meteorological stations considered, median value in the interval 2003-2013; Themeßl et al. 2011; ARPAV 2014), and the resolution was fixed in a 250 m x 250 m regular grid.

Together with winter temperature, annual rainfall is often considered a key parameter regulating the presence of *Phytophthora* spp. in chestnut stands, and precipitation greater than 1000 mm/year is considered a useful index to classify areas at risk for CID (Vettraino et al., 2005; Vannini et al., 2012). Therefore, the annual rainfall map was downloaded from the BIOCLIM database (BIO12; Beaumont et al., 2005), downscaled and corrected as described above for the previous environmental predictor.

In terms of summer climatic conditions, limited droughts do not influence the survival of *Phytophthora* spp. However, following fine feeder root infections, periods of drought can lead to dieback or sudden collapse (Delatour, 2003; Johnston et al.

2003; Jung et al. 2003; Vettraino et al. 2005; Scanu et al. 2013; Corcobado et al. 2014). Giacobbe's index was consequently included in the model as a summer drought index (Giacobbe, 1967; Gavilán, 2005). The maps necessary for its computation (in particular, June, July and August precipitation and the maximum temperature of the warmest month) were acquired from the WORLDCLIM and BIOCLIM databases (Beaumont et al. 2005) and were downscaled and corrected as described above.

In terms of the effects of geographical features, Vannini et al. (2010) have indicated that CID severity is significantly higher in proximity to natural sources of surface water drainage that lead to flooding or soil saturation events. This relationship has also been highlighted by Brasier (1996) and Corcobado et al. (2013) for *P. cinnamomi* infections on *Quercus* spp. Therefore, a stream map of the investigated area was used (Regione del Veneto, Dipartimento Difesa del suolo e foreste, internal report), assuming a value of 0 for every grid cell that contained a stream and a value of 1 for every grid cell that did not contain a stream but was adjacent to a cell containing a stream. Every grid cell that was n>1 cells away from the nearest cell containing a stream was assigned a value of "n".

Slope aspect is another factor that is commonly thought to influence CID incidence because infected chestnut are more frequently found in south-facing stands (Brasier, 1996; Portela et al., 1999; Vannini and Vettraino, 2001; Marçais et al., 2004). The "Northness" index, which quantifies the degree to which a slope has a northerly aspect, was therefore calculated with the following formula:

$$Northness = cos\left(\frac{aspect\ \ in\ \ degrees \times pi}{180}\right)$$

For example, the northness index for an angle of 360 degrees (indicating north) is equal to 1, for 90 degrees (equal both to east and to west) is 0, and for 180 degrees (towards the south) is –1 (Morrison et al., 2003; Molotoch et al., 2005). To calculate northness, aspect was processed in Arcmap v. 10.2 (ESRI, 2014) using ASTER Global Digital Elevation Model data v. 2 (~30 m resolution; NASA Land Processes Distributed Active Archive Center (LP DAAC), 2011), and the final northness index value for each grid cell corresponded to the median of the contained values.

Edaphic factors are usually included in the CID reference frame, and incidence was shown to increase with compaction (Martins et al., 1998; Vannini and Vettraino, 2001; Fonseca et al., 2004, Martins et al., 2007). Because compaction is difficult to assess in large areas and at large scales, and as permeability is an influential factor favouring root rot caused by *Phytophthora* spp. (Bounous, 2006), only permeability was considered. The official soil permeability map that was used had a scale of 1:50000 (Provincia di Treviso and ARPAV, 2008). Nominal classes were transformed to reference values (Table 9) to allow the computation of the weighted average on the basis of the per cent coverage in each cell.

Because various authors recommend considering soil reaction (soil pH) as an important factor influencing *Phytophthora* spp. presence (Jung et al., 2000; Vettraino et al., 2005; Brasier et al., 2009; Jönsson, 2006), a soil reaction map was also used (Provincia di Treviso and ARPAV, 2008).

Finally, soil organic matter content has been reported to be inversely proportional to CID incidence (Portela et al., 1999; Vannini and Vettraino, 2001; Vannini et al., 2010). In contrast, Fonseca et al. (2004) observed that an increase in organic matter increased the probability that a stand would develop CID because the pathogens could even benefit from it due to their poor saprotrophic nature (McCarren et al., 2005; Acosta-Muñiz et al., 2012). Given the contradictory

Permeability class	**Ksat (µm/s)**	**Reference value**
From low to very low	< 0.01	0
Low	0.01 - 0.1	12.5
From low to moderately low	0.1 - 1	25
Moderately low	1 - 10	37.5
From moderately low to moderately high	10 - 100	50
Moderately high	100 - 1000	62.5
From moderately high to high	1000 - 10000	75
High	10000 - 100000	87.5
From high to very high	> 100000	100

Table 9. Reference values associated with the nominal classes of soil permeability (Provincia di Treviso and ARPAV 2008) to extract an average value for each grid cell of the study area. Ksat indicates the saturated hydraulic conductivity, defined as the ability of a soil to conduct water under saturated conditions (Blanco-Canqui and Lal, 2008).

information in the literature, this variable was not integrated into the model.

All the environmental maps were analysed in Arcmap v. 10.2 (ESRI, 2014) to assess the ranges of the predictors covered in the study area.

Fuzzy model construction and uncertainty computation

To implement fuzzy set theory into the model, the fuzzy logic toolbox from MATLAB v. 8.3 (MATLAB, 2014) was used. Fuzzy sets were used to describe the five final ecological variables considered (minimum temperature of the coldest month, Giacobbe's index, stream distance, northness index and soil permeability). Two trapezoid membership functions ("low" and "high"; Van Broekhoven et al., 2006; Mouton et al., 2009b) characterised by four parameters (a, b, c, d) were created for each variable (Table 10). The degree by which a single variable value belonged to a membership function increased linearly from 0 to 1 if the variable had a value in the range [a, b], was equal to 1 in [b, c], and decreased linearly from 1 to 0 in [c, d]. For instance, if the soil permeability was 57 %, the value belonged to "low" function soil permeability with a degree of 0.85 and to "high" with a degree of 0.15 (Figure 15).

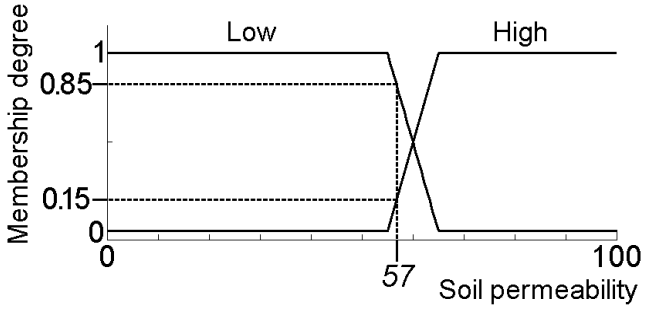

Figure 15. Schematic illustration of the central concept of fuzzy logic. The two horizontal lines indicate how the same input value is a partial member of both the "low" permeability class and the "high" permeability class.

The fuzzy rule-based model related the input membership functions to habitat suitability for the two *Phytophthora* species and consisted of if-then rules, such as "IF Minimum temperature is low AND Giacobbe's index is low AND Stream distance is high AND Northness index is low AND Soil permeability is high THEN habitat suitability IS low". The information included in the knowledge base was, therefore, summarised into 32 rules to be used for both *Phytophthora* species (Table 11).

In the current application, the fuzzy AND ('min') operator was used for implication, and the fuzzy OR ('max') operator was used for aggregation in the Mamdani-Assilian inference method (Mamdani and Assilian, 1975). The fuzzy output was then defuzzificated with the centre-of-gravity formula (Adriaenssens et al., 2004), obtaining a crisp output value in the range [0, 1] (from very low to high suitable context).

The FM was run for every grid cell of the study area in Simulink v. 8.3 (MATLAB, 2014) and projected by means of the Arcmap platform (ESRI, 2014); then the map was exported in Keyhole Markup Language ("kml") format to allow the visualization in Google Earth (Google Earth, 2014) for end users.

Input variables	**Low [a b c d]**	**High [a b c d]**
Minimum temperature of the coldest month (°C)	trapmf [-15 -15 -4 -3] ± 2	trapmf [-4 -3 5 5] ± 2
Giacobbe's index (n)	trapmf [0 0 11.5 12] ± 2	trapmf [11.5 12 23 23] ± 2
Stream distance (n)	trapmf [0 0 0.5 1] ± 1	trapmf [0.5 1 20 20] ± 1
Northness index (n)	trapmf [-1 -1 -0.1 0.2] ± 0.2	trapmf [-0.2 0.1 1 1] ± 0.2
Soil permeability (%)	trapmf [0 0 55 65] ± 10	trapmf [55 65 100 100] ± 10

Output variable	**Very low [a b c d]**	**Low [a b c d]**	**Intermediate [a b c d]**	**High [a b c d]**	**Very high [a b c d]**
Prediction (Habitat suitability)	[0 0 0.15 0.25]	[0.15 0.25 0.35 0.45]	[0.35 0.45 0.55 0.65]	[0.55 0.65 0.75 0.85]	[0.75 0.85 1 1]

Table 10. Membership functions for input and output variables used in the fuzzy model. Functions are explained by means of the characterising nodes (a,b,c,d) of the trapmf (trapezoidal) function; the range of variation for uncertainty analysis is given under each set.

Table 11 (*in the following page*). Fuzzy rule-based system for inferring habitat suitability for chestnut ink disease with input variables.

Minimum temperature	Giacobbe's index	Stream distance	Northness index	Soil permeability	Habitat suitability for CID
Low	Low	Low	Low	Low	Medium
Low	Low	Low	Low	High	Low
Low	Low	Low	High	Low	Medium
Low	Low	Low	High	High	Low
Low	Low	High	Low	Low	Medium
Low	Low	High	Low	High	Low
Low	Low	High	High	Low	Medium
Low	Low	High	High	High	Low
Low	High	Low	Low	Low	Low
Low	High	Low	Low	High	Very low
Low	High	Low	High	Low	Low
Low	High	Low	High	High	Very low
Low	High	High	Low	Low	Low
Low	High	High	Low	High	Very low
Low	High	High	High	Low	Low
Low	High	High	High	High	Very low
High	Low	Low	Low	Low	Very high
High	Low	Low	Low	High	High
High	Low	Low	High	Low	Very high
High	Low	Low	High	High	High
High	Low	High	Low	Low	Very high
High	Low	High	Low	High	High
High	Low	High	High	Low	Very high
High	Low	High	High	High	Medium
High	High	Low	Low	Low	High
High	High	Low	Low	High	Medium
High	High	Low	High	Low	High
High	High	Low	High	High	Medium
High	High	High	Low	Low	High
High	High	High	Low	High	Medium
High	High	High	High	Low	High
High	High	High	High	High	Low

To evaluate the usefulness of model results, the framework of uncertainty provided by Walker et al. (2003) was used, and the computations reflected every type of uncertainty occurring in this type of modelling, as detailed below:

- Model structure uncertainty, linked to the impreciseness of knowledge related to the structure itself, was assessed as the difference between the left and right centres of area in the defuzzification process (Refsgaard et al., 2007; Janssen et al., 2010).
- Input uncertainty comprises external forces of a stochastic nature due to the variability in the system (Refsgaard et al., 2007; Walker et al., 2003). For each case in the rules table (Table 11) and all the intermediate combinations, a Monte Carlo analysis (n = 10000) was run on the inputs, for which a normal probability distribution with a standard deviation equalling 30 % of the reference value was assumed (Table 10; Janssen et al., 2010). For each case, the 25-75 percentile range was computed.
- Parameter uncertainty is associated with the data and the methods used to calibrate the model parameters (Walker et al., 2003). Model propagation of this type of uncertainty was evaluated with a sensitivity analysis according to Janssen et al. (2010; ranges for parameters are given in Table 10), obtaining the 25-75 percentile range of the differences from the defuzzified model's outputs.

Monitoring and model validation

The data for the model validation were acquired in a detailed phytosanitary field survey in August and September of 2014, during which 100 sites were investigated for CID presence (Figure 14). To consider only comparable locations with at least 10 chestnuts, monitoring points were randomly chosen in the chestnut stands in the forest map previously described (Del Favero et al., 2000; Regione del Veneto, 2006), and the position was locally controlled by means of a GPS tool with 4±1 m resolution (model GSMAP62sc, Garmin Ltd., Southampton, Hampshire, UK). At each site, when at least one tree showed symptoms attributable, also in part, to CID (Figure 16; Vannini and Vettraino 2001; Vettraino et al. 2005; Prospero et al. 2013), 10 subcortical samples (~ 15 cm^3) were collected from both structural roots (5 samples) and the trunk base (5 samples) for each of 5 alive chestnuts. When less than 5 symptomatic trees were available, the number was reached sampling chestnuts neighbouring to symptomatic ones. All of the specimens were gathered together, carefully mixed and immediately processed twice by means of a lateral

Figure 16. Different classes symptoms of ink disease, from healthy chestnut (on the left) to high symptomatic ones (on the right).

flow test (Pocket Diagnostic test kit for the detection of *Phytophthora*, Forsite Diagnostics Ltd., Surrey, UK) to confirm the presence of the genus. If a site was associated with symptomatic chestnuts and positive results from the lateral flow test, it was classified as "positive".

If all of the chestnuts in the cell were asymptomatic, basal woody samples were collected from randomly chosen trees and processed through lateral flow tests as above reported, to confirm the "negative" status of the cell.

Based on the contingency table, validation of the model's predictive results was conducted for overall accuracy, specificity, sensitivity, Correctly Classified Instances (CCI), Cohen's Kappa statistic (K), the True Skill Statistic (TSS) and F_{pb} index, with the latter specifically relying on presences and pseudoabsences (Table 12; Liu et al. 2005, Allouche et al., 2006; Li and Guo, 2013; Maddock et al., 2013). A model output was considered "positive" if the predicted output had a value greater than 0.5 in a range [0, 1] (Adriaenssens et al., 2006; Li and Guo, 2013). Finally, the Area Under the Curve (AUC) of the receiver operating characteristic (ROC; Manel et al., 2001; Brotons et al., 2004; Thuiller et al., 2005; Peterson et al., 2008) was obtained in IBM SPSS Statistics software (v. 22, International Business Machines Corp., New York, USA; Elith, 2000).

Measure	Formula
Overall accuracy	$\frac{TP+TN}{n}$
Sensitivity	$\frac{TP}{TP+FN}$
Specificity	$\frac{TN}{TN+FP}$
Kappa statistic	$\frac{\left(\frac{TP+TN}{n}\right)-\frac{\left(TP+FP\right)\left(TP+FN\right)+\left(FN+TN\right)\left(TN+FP\right)}{n^2}}{1-\frac{\left(TP+FP\right)\left(TP+FN\right)+\left(FN+TN\right)\left(TN+FP\right)}{n^2}}$
TSS	$Sensitivity + Specificity - 1$
F_{pb}	$\frac{2\times TP}{TP+FN+FP}$

Table 12. Parameters used in the evaluation of fuzzy model. Abbreviations: TSS, True Skill Statistic; CCI, Correctly Classified Instances; n, total number of cases; TN, true negative; FP, false positive; TP, true positive; FN, false negative.

Results

Environmental predictors choice

After analysing the possible environmental predictors projected in the study area, the final chosen input variables were as follows: minimum temperature of the coldest month, Giacobbe's index, stream distance, northness index and soil permeability. Annual rainfall and soil pH were discarded from further analyses because every grid cell had values higher than the suggested thresholds (>1000 mm and > pH 4, respectively).

Fuzzy model prediction and uncertainty computation

When the model outputs were evaluated, the rule view interactive interface enabled access to individual output values of habitat suitability according to the input values of the chosen environmental predictors (see Figure 17 for an example). The results of the fuzzy simulations were summarised by calculating the habitat

suitability surface for the study area (Figure 18), and the computed suitability scores (p) fell within the [0.09, 0.9] interval in the possible suitability score range [0, 1]. The greater (central-western and southern) part of the study area was covered by high and very high probabilities (36.5 % coverage for p in [0.5, 0.75], 35 % for p in [0.75, 1], respectively), indicating high suitability for development of CID. In contrast, the areas associated with low and very low predictions (18.8 % coverage for p in [0.25, 0.50], 9.7 % for p in [0, 0.25], respectively) were located in higher-elevation zones, restricted to the northern and eastern areas.

The depicted uncertainty maps indicate the variability of predictions on the basis of different simulations. Model structure uncertainty (Figure 19), referring to the variability due to the interrelations among the elements of the system, was low (min 0.09, max 0.19, mean 0.13, median 0.15), considering the possible range [0, 1], and homogeneously distributed (SD 0.028). In contrast, the input uncertainty (Figure 20) revealed that a small stochastic variation in the input parameters could lead to a significant variation in the prediction of the transitional zones between "positive" and "negative" areas. The statistics indicated that the input uncertainty varied from a minimum of 0 to a maximum of 0.37 (mean 0.058, SD 0.1, median 0). Finally, parameter uncertainty (Figure 21), related to *a priori* chosen parameters defining the membership functions, was low in nearly the entire study area (min 0, max 0.18, mean 0.05, SD 0.037, median 0), considering the achievable range [0, 1].

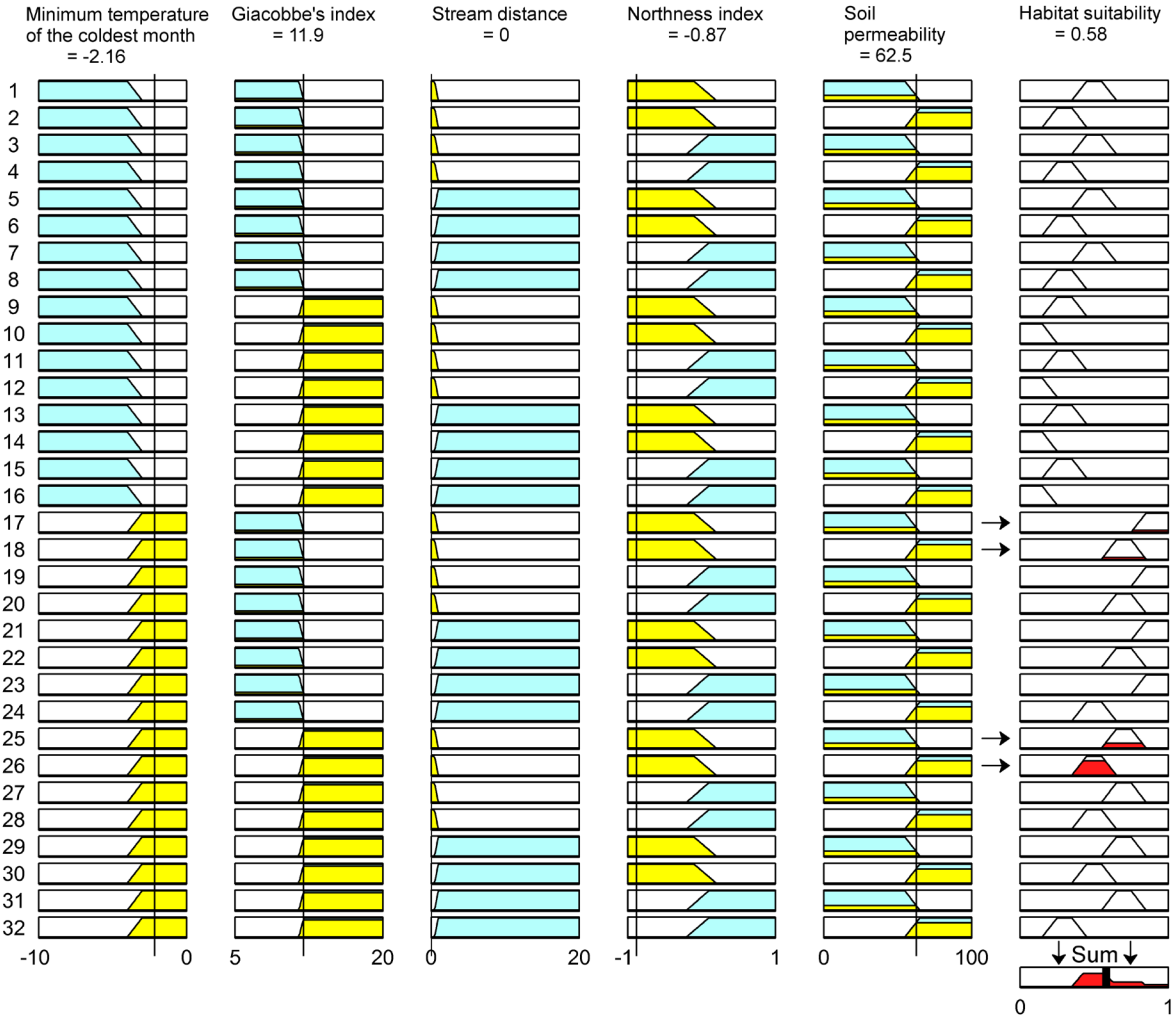

Figure 17. Rule view interface used to access individual output values of habitat suitability according to input values. In this example, input values are reported in the column labels and depicted as vertical lines in the underlying plots. Each rule (reported in Table 11) is explained as a row of plots and each column is a variable. The first five columns show the membership functions for the if-part of each rule: in each plot, satisfied membership functions for the considered input are reported in yellow, unsatisfied ones in blue. The sixth column shows the membership functions referenced by the then-part of each rule (Table 11) and the bottom plot represents the aggregate weighted decision for the given inference system, where the defuzzified output is displayed as a bold vertical line.

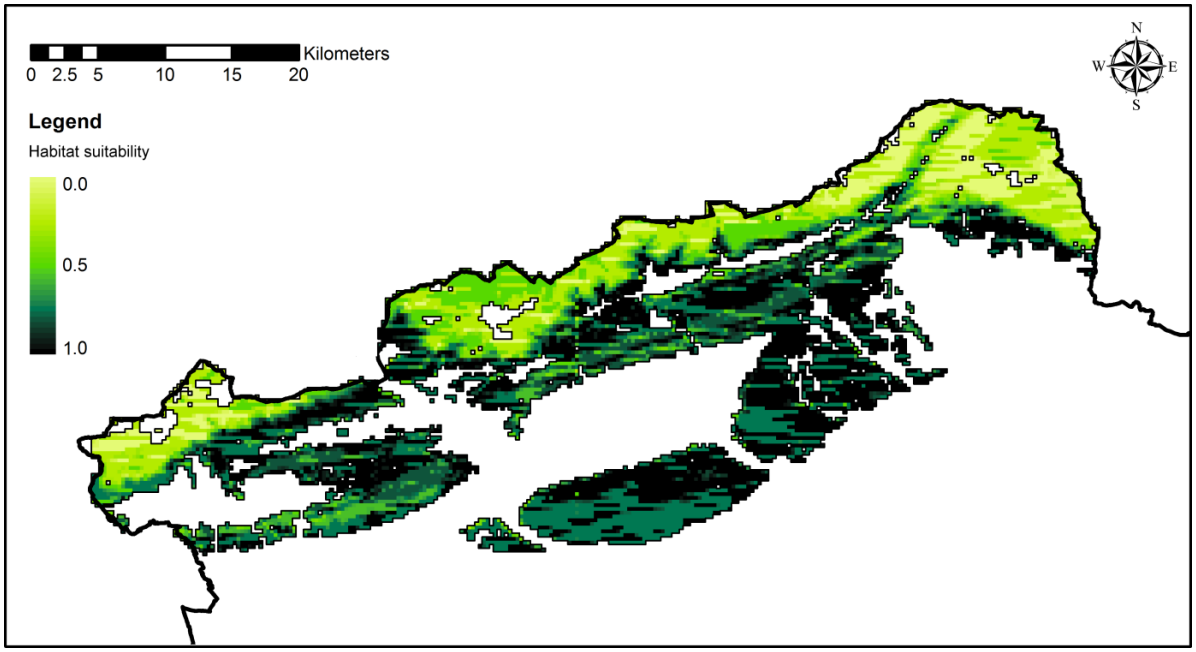

Figure 18. Predicted spatial habitat suitability for chestnut ink disease in the study area. Different colours represent different levels of predicted probability.

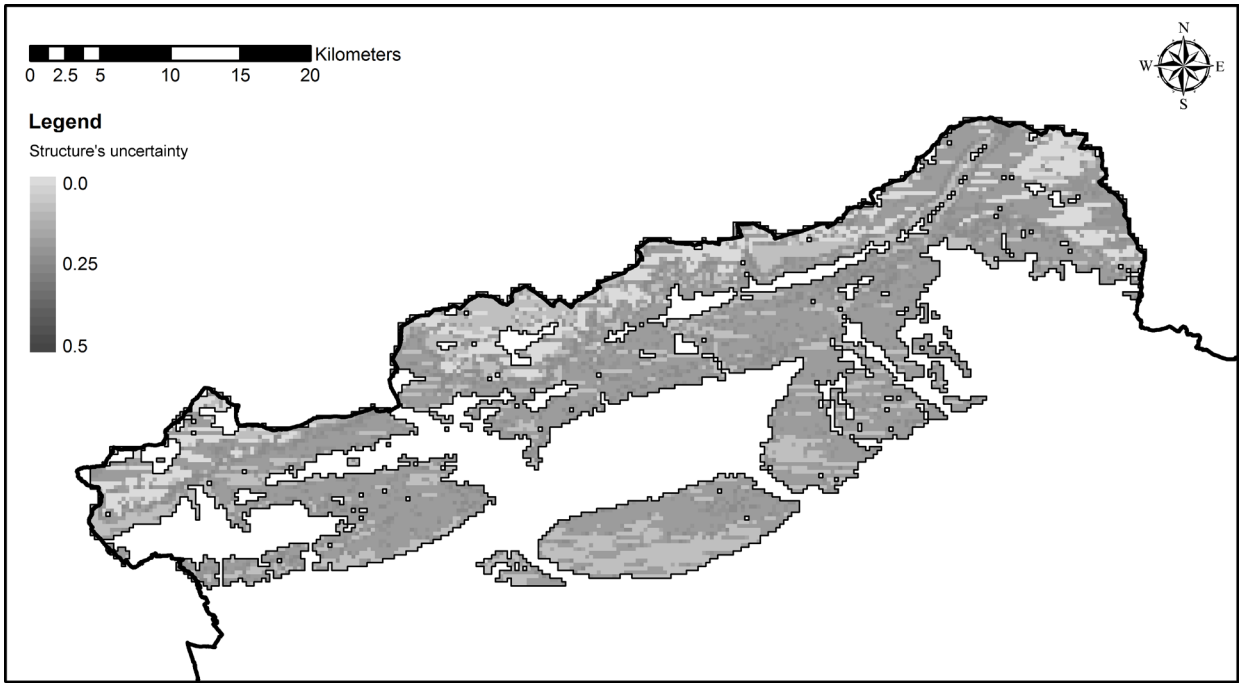

Figure 19. Outcomes of the propagation of the model structure uncertainty for the study area. Values denote the difference bewteen 75 and 25 percentile values around the median.

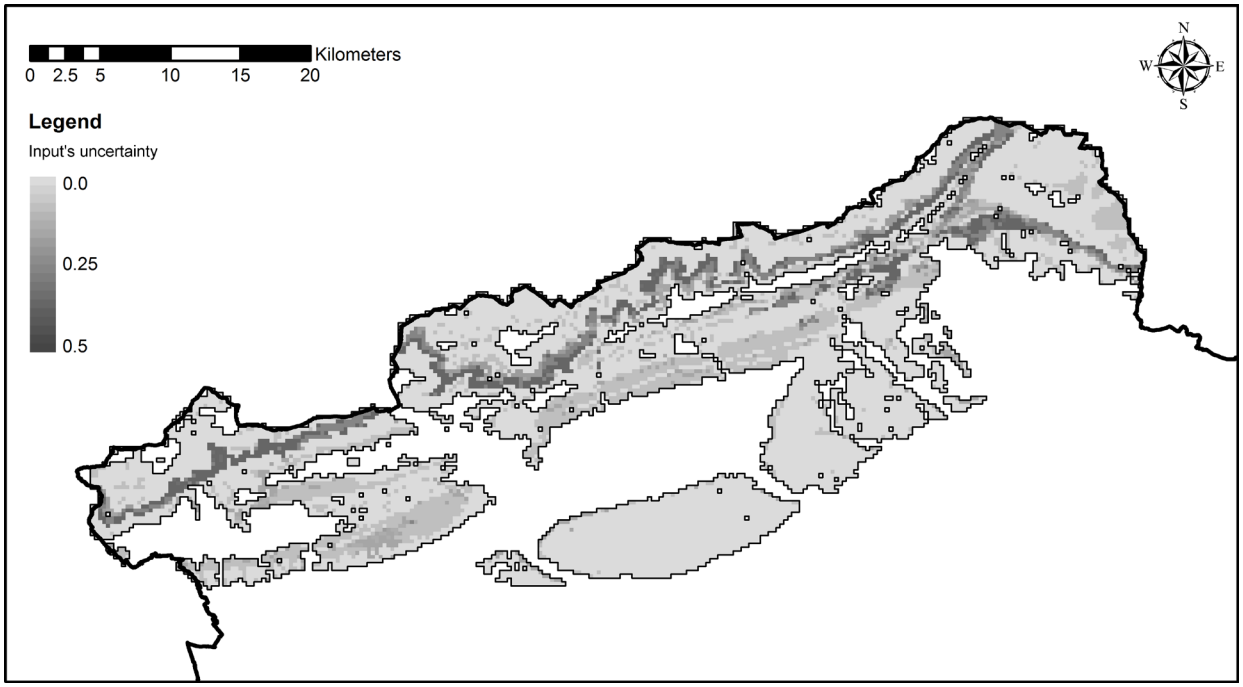

Figure 20. Outcomes of the propagation of the input uncertainty for the study area. Values denote the difference bewteen 75 and 25 percentile values around the median.

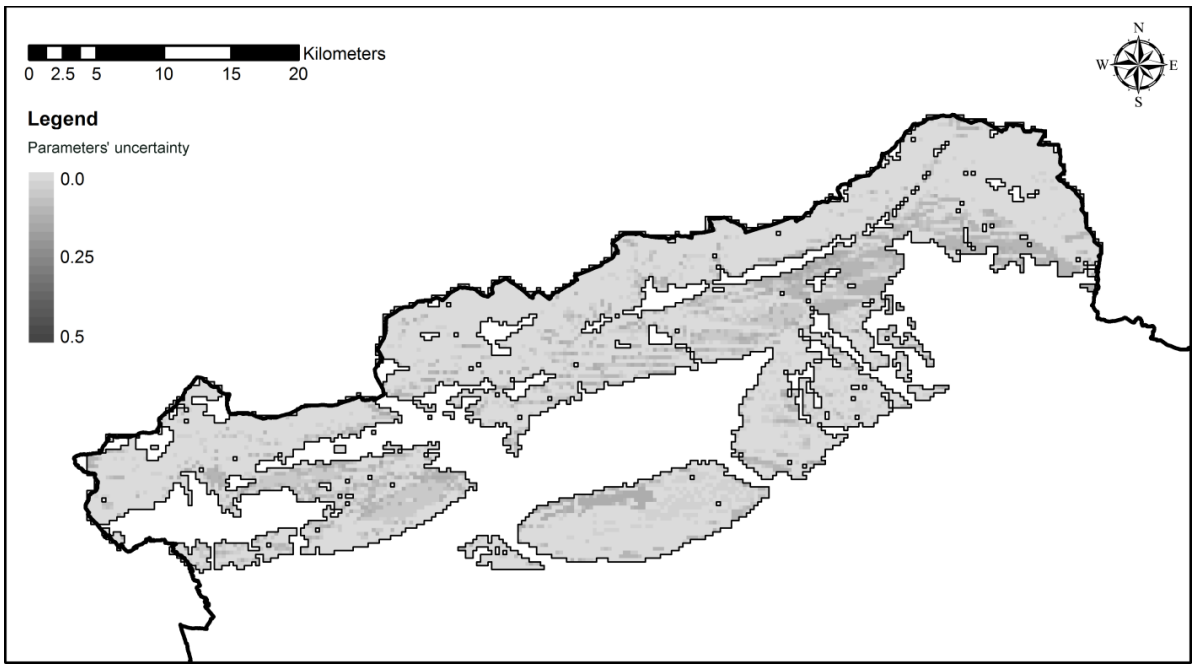

Figure 21. Outcomes of the propagation of parameter uncertainty. Values denote the difference bewteen 75 and 25 percentile values around the median.

Monitoring and fuzzy rule-based model validation

According to the field surveys conducted to validate the model, According to the field surveys conducted to validate the model, the presence of *Phytophthora* genus was confirmed by lateral flow devices in all sites with symptomatic trees (59 positive cells). Lateral flow's results were negative in all the sites with asymptomatic chestnuts (41 negative cells).

A contingency table was created combining the predicted probabilities (threshold = 0.5) and corresponding monitoring data (Table 13). The true positive rate (TP) was 54 % and the true negative rate (TN) was 21 %; in contrast, the false positive rate (FP) was 20 % and, overall, the false negative (FN) rate was 5 %.

		Monitoring data		
		Negative	**Positive**	**Total amounts**
Model prediction	**Negative**	21	5	26
	Positive	20	54	74
Total amounts		41	59	*100*

Table 13. Contingency table (also called confusion matrix) combining the fuzzy model predictions and monitoring data. Being 100 the total number of surveyed points, the reported percentages correspond to real values. Total amounts for positives and negatives for model predictions and monitoring data are reported in the right column and bottom row respectively, and grand total is in italic.

The resulting performance measures are reported in Table 14, showing that model evaluations revealed medium performance for overall accuracy (corresponding to the Correctly Classified Instances, CCI), specificity, Cohen's Kappa statistic (K), the True Skill Statistic (TSS) and the Area Under the Curve (AUC) of the receiver operating characteristic (ROC). In the context of the data set used for evaluation, these values were greatly reduced by the contribution of the false positive rate. If the measures that specifically rely on presences and pseudoabsences (Table 12, Table 14) are considered, the performance of the method for sensitivity and the F_{pb} index was very good.

Evaluation parameter	Value
Overall accuracy (CCI)	0.75
Sensitivity	0.91
Specificity	0.51
Kappa statistic	0.45
TSS	0.43
F_{pb}	1.37
AUC	0.74

Table 14. Fuzzy model performance calculated on the basis of monitoring data acquired in the study area. AUC represents the Area Under the Curve of the receiver operating characteristic (ROC); in Table 12 the explanation for the other performance's measures is reported.

Discussion

The present study incorporated available environmental information on CID into a new inference system for habitat suitability modelling by expressing non-linear relationships in terms of if-then rules (computing with words; Adrianssens et al., 2006; Van Broekhoven et al., 2006; Fukuda, 2009), then graphically transposing them into the final thematic map. For instance, CID habitat suitability for all of the 10567 considered cells was calculated as "very high" when the site was characterised by a high winter minimum temperature, a high probability of summer drought, presence of streams, a southerly aspect and a low soil permeability.

As usual, the key sources of uncertainty in presence and prediction maps are model structure, input data and parameter values (Refsgaard et al., 2006), with an optimal model able to provide the greatest simplifications together with an accurate representation of the investigated phenomenon. From this point of view, model structure uncertainty arises from the approximation of the physical and biological world by mathematical expressions (Ascough et al., 2008), and addressing the level of uncertainty for the correct interpretation of outcomes and results is essential (Loucks et al., 2005; Janssen et al., 2010). The structure uncertainty map for the current fuzzy rule-based model showed very low

uncertainty in the study area, and a slight variation in the model's parameters did not noticeably change the final prediction, indicating the robustness of the model system (Mayne et al., 2000). As noted by Kim and Beresford (2012), input surfaces obtained from spatial interpolation may contain uncertainty and imprecision, due also to the inherent randomness of nature (i.e., the chaotic and unpredictable quality of natural processes; Ascough et al., 2008). In this respect, the obtained input uncertainty map showed that a fluctuation in the input values tended to modify predictions in the transitional zones between high and low suitability areas, which, on the contrary, were little affected.

The developed FM was validated using a significant amount of data directly collected in the study area considering CID symptoms and *Phytophthora* genus-specific lateral flow devices outcomes. The comparison of survey's results with model predictions demonstrated the need to consider various performance criteria. Specifically, overall accuracy, specificity, the Kappa statistic, TSS values and AUC reflected moderate to good performance by the model. In particular, moderate outcomes were derived from the evident number of false positive observations. Effectively, K_{pb} and sensitivity measures, which address these types of data (Li and Guo, 2013), were substantially higher. Previous research has shown that, from an ecological point of view, models that overpredict observations may not necessarily be ecologically irrelevant (Mouton et al., 2009a, 2011). Underpredictions (false negative observations) always imply a model error, whereas overpredictions (false positive points) may be due to unbalanced colonisation of suitable habitats in the considered area (Mouton et al., 2011). In the specific case of CID, overpredictions could be caused, for instance, by physical barriers separating disjoint areas, consistent with the property that soil-borne pathogens depend on animals and waterways for long-range dispersal (Vannini et al., 2010; Gonthier and Nicolotti, 2013). However, the underpredictions (false negative observations) detected in the validation procedure were included in transitional areas with the highest input uncertainty.

The FM was demonstrated to be simple (relations between input and output are explained with linguistic rules) and robust (performance does not depend on the training data; Adrianssens et al., 2004, 2006). Moreover, this type of model is a flexible tool, as it can be easily updated with new input variables and rules. For instance, the annual rainfall predictor can be included at a later time, or previously

considered variables can be discarded when applying the model to different geographical situations. Moreover, thanks to its linguistic aspect, the FM model and its results are transparent and interpretable by a wide range of end users, facilitating communication among modellers, ecologists and forest managers (Adrianssens et al., 2004; Chen and Mynett, 2003).

The model described here, employed in forest pathology for the first time, may be a useful decision support tool in pest risk management. Effectively, ranking a current or potential chestnut area for the probability of CID presence can allow for better forest conservation strategies (Vannini et al., 2010). The accurate geographical identification of transitional zones (corresponding to the border of infected and symptomatic areas) could facilitate focused containment measures along an outbreak frontline (Bounous and Abreu, 1998; Brasier, 1999; Gentile et al., 2010). In particular, integrated pest management (IPM) strategies could be attempted by 1) potassium phosphite treatments through trunk injection (Gentile et al. 2009; Vannini et al. 2009), 2) chicken manure distribution (Turchetti and Maresi 2006, 2009) and 3) soil moisture drainage (Gentile et al. 2009). As a subsequent step, a software application based on the developed fuzzy logic model and requiring additional information such as the susceptibility of host populations (Robin et al., 2006) could be realised for a more detailed and site-specific approach.

CHAPTER V

Efficacy of potassium phosphite formulations against chestnut ink disease by trunk injection

СРАВНИТЕЛЬНЫЕ ИСПЫТАНИЯ ИНЪЕКЦИИ В СТВОЛ ЧЕТЫРЕХ КОМПОЗИЦИЙ КАЛИЯ ФОСФИТА ПРОТИВЧЕРНИЛЬНОЙ БОЛЕЗНИ КАШТАНОВ

Proceedings to:
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Abstract

Ink disease, caused by *Phytophthora cinnamomi* and *P. cambivora*, is one of the most destructive diseases affecting *Castanea sativa*. Currently, disease control requires careful integrated chemical and agronomic measures. Trunk injection with potassium phosphite was shown as curative in reducing symptoms expression but little is known about the ideal formulation and potential adjuvants. A preliminary endotherapic trial was conducted in a chestnut where *P. cinnamomi* was isolated, with the main aim to evaluate growth stimulation of active growing callus next to the shape flame necroses, by the injected solution of potassium phosphite 70 %. In this case, results did not highlight a significant difference between treated trees and water control ones. In a further research, fifty asymptomatic sweet chestnuts were inoculated with *P. cinnamomi*. Subsequently, trees were injected with four formulations of potassium phosphite. In comparison with water treatment, after 50 days the growth of the necroses was significantly slowed down only by one formulation, consisting in potassium phosphite added with a micronutrient solution. The results increases the knowledge base on the efficacy of endotherapy against chestnut ink disease.

резюме

Чернильная болезнь, вызвана *Phytophthora cinnamomi* и *P. cambivora*, является одним из самых разрушительных болезней, поражающихсъедобный каштан *(Castanea sativa).* В настоящее время, борьба с болезнями, требует тщательных комплексных химических и агрономических мер.По доступной литературе, инъекциикалия фосфита в ствол снижает экспрессию симптомов, но мало данных об идеальной постановке и потенциальных вспомогательных веществ.

В этом исследовании, пятьдесят здоровых каштанов были привиты с *P. cinnamomi*, локально изолированы от симптоматических каштанов.Двадцать дней спустя, в деревья были сделаны инъекции четырех составов, содержащих фосфит калия.По сравнению с применении воды, после 50 дней рост некрозов была значительно замедлена только с одним препаратам, состоящего из фосфита калия с добавленнымимикроэлементами.

Результаты этого исследования, вместе с некоторыми техническими предложениями для применения в практике, поддерживают идею использования endotherapicпродуктов для содержания заболевании, с безопасным и очень низким влиянием метода инъекции в ствол.

Introduction

Phytophthora cambivora (Petri) Buism. and *P. cinnamomi* Rands are soil-borne pathogens responsible of the so-called chestnut ink disease (CID), one the most destructive diseases of sweet chestnut (*Castanea sativa* Mill; Vannini and Vettraino, 2001; Vettraino et al., 2005; Choupina et al., 2014). CID symptoms comprise dieback of the distal branches, defoliation, root and collar necroses, tannic fluid leaks, gradual decline and host death (Vannini and Vettraino, 2001; Vettraino et al., 2005; Vannini et al., 2010; Prospero et al., 2013). Chestnuts' loss entailed not only an economical and cultural damage, but it compromises also the stability of slopes or ridges, leaving them exposed to erosion from runoff rainwater (Maresi and Turchetti, 2008).

Chestnut ink disease can be prevented or controlled by integrated chemical and agronomic measures and protocols, that imply, for instance, management of water's flows and fertilizing (IPC; Bounous and Abreu, 1998; Brasier, 1999; Gentile et al., 2010). Among compounds employed in CID chemical control, metalaxyl and copper compounds represent efficient solutions against *Phytophthora* spp., but were proved to deliver toxic impact on micobiont when applied to the soil (Graham et al., 1986) or insurgence of resistant isolates (Franceschini, 2011), respectively. Phosphonates are effectives both *in vitro* and *in planta* against *P. cinnamomi* and *P. cambivora* (Coelho et al., 2005; Hardy et al., 2001; Wilkinson et al., 2001; Gouveia et al., 2010), acting directly at high concentration or stimulating host defence at low concentration (Jackson et al., 2000). In comparison with phosphite foliar treatments (Pilbeam et al., 2000; Hardy et al., 2001), trunk injection can lead to less or none phytotoxic effect, varying considerably with the dose and at a family and genus level (Garbelotto et al., 2007). Phosphite trunk injections was proved effective against *Phytophthora cinnamomi* in Avocado and in chestnuts (Darvas et al., 1984; Gentile et al., 2009), but little is known about ideal concentration and potential adjuvants.

The aim of this study was to ascertain best performing formulates to control CID in artificially infected trees by trunk injections (Tattar et al., 1998; Takai et al., 2003; Aćimović et al., 2014). Furthermore in planta test on symptomatic chestnuts were conducted to evaluate recovering of chestnuts treated with high concentration potassium phosphite on the basis of growing increase of active

growing callus.

Materials and Methods

Measurement of callus growth after potassium phosphite treatment

The research was performed in a chestnut in the Veneto region (Northeastern Italy) where, after a first report at regional scale in 1923 (Petri, 1923), ink disease was observed in 2007 (Scattolin et al., 2012). The site (45° 47 ′ N; 11° 50 ′ E; 110 – 150 m a.s.l.) is a private, unmanaged sweet chestnut forest characterized by wet temperate climate with warm summers, annual mean temperature of 12.5° C, equinoctial rainfall regimen with two maxima in spring and autumn (266 and 264 mm, respectively) and mean annual rainfall of 705 mm. The soil is classified as Acrisol-Alisol, according to FAO-UNESCO-WRB (FAO-WRB, 1998). The study plot is approximately 4-ha wide, with a 25 – 40 % slope. The vegetation type is typical of the Castanetum (De Philippis, 1937), with > 10 % of *Fraxinus excelsior* L., *Robinia pseudoacacia* L. and *Corylus avellana* L.

Due to the need for alive trees with similar ink disease symptoms and, in particular, with shape flame necroses at the collar, after careful selection 14 chestnuts were chosen for the experiment, ranging from 18 to 47 cm (ave. 30 cm) at breast height (dbh). For each tree, 10 subcortical samples (~ 15 cm^3) were collected from both structural roots and trunk. The specimens were immediately processed by means of a lateral flow test (Pocket Diagnostic test kit for the detection of *Phytophthora*, Forsite Diagnostics Ltd., Surrey, UK) to confirm the genus presence. Moreover, soils cores (10 x 20 cm, 45 cm in depth) were collected radially from the stem base (50 cm from the trunk) along the maximum slope direction (up or down) and along its perpendicular direction (Iso), 30 cm far from the collar. The samples were processed by baiting (Jung et al., 1996; Franceschini, 2011) using fresh *C. sativa* leaves as baits. Baiting assays were kept at room temperature until the development of necroses (5-10 days). Baits were then gently cleaned under running water for 1 hour and let dry on sterile paper. Small portions (3 x 3 mm) of fresh active lesions, where the necrotic tissues are continuous with healthy tissues, were plated on PDA (Potato Dextrose Agar, Difco Laboratories, Detroit, MI, USA) in 60 mm diam. Petri dishes and incubated at 24 ± 1° C in the dark and daily checked for fungal cultures with coenocytic mycelium, immediately sub-cultured on PDA. *Phytophthora*-like colonies, presenting characteristic colony

pattern and mycelium (Erwin and Ribeiro, 1996; Scanu et al., 2014), but not producing sporangia, were treated according to Halsall and Forester (1977), in order to allow the production of fructification. The measurements of sporangia and chlamydospores allowed a first species identification (Erwin and Ribeiro, 1996), in order to select the cultures for molecular identification processing.

Colony PCR was directly applied on a representative isolate without DNA extraction according to Kong et al. (2005), together with an official strain of *P. cinnamomi* (CBS 144.22; CBS-KNAW Database, www.cbs.knaw.nl/). In particular, cytochrome oxidase genes encoding subunits I (*cox1*) primers (FM84 TTTAATTTTTAGTGCTTTTGC and FM83 CTCCAATAAAAAATAACCAAAAATG for amplification; FM50 GTTTACTGTTGGTTTAGATG for sequencing; Martin et al., 2014) were used. The chosen thermocycling pattern comprised an initial denaturation at 95° C for 3 min, followed by 35 cycles of 95° C (1 min), 56° C (1 min) and 72° C (2 min), and a final extension step of 72° C for 5 min. A sample of 10 µl of the PCR product was electrophoresed on a 1 % agarose gel together with MassRuler DNA Ladder Mix (#SM0403; Thermo Scientific, MA, US), visualized by staining with Green Gel Plus™ (Fisher Molecular Biology, Società Italiana chimici distributor, Rome) and imaged using a UVIpro Gold Gel Documentation System (UVItec, Cambridge, UK). The amplified products were first purified by an enzymatic reaction using Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA) and then sequenced by BMR Genomics (Padua, Italy) using Sanger sequencing methodology. The sequences were compared with reference ones in the NCBI (http://www.ncbi.nih.gov/BLAST; Benson et al., 1999) using the Basic Local Alignment Search Total nucleotide search (BLASTn) program (Altschul et al., 1997). Sequence taxon categories were assigned as follows (Bidartondo and Read, 2008): sequence similarity of 99 %, identification to the species level; sequence similarity of 95 – 99 %, identification to the genus level; and sequence similarity of 95 %, identification to the family level.

According to both tree diameter and the percentage of necrotic areas at the collar, selected chestnuts were split in two comparable groups (Peterson et al., 2009), to be injected with commercial formulations of potassium phosphite 70 % (Agrofill by Adriatica S.p.a.; 21 % phosphoric acid and 14 % potassium oxide final concentrations) and water, as a control. In May-June 2014, trees were injected at

breast height in 4-6 equidistant points with 3 mL/cm dbh of each solution with a handheld tool recently developed by the University of Padova (BITE; Montecchio, 2013). At the same time, 15 nails were carefully inserted in the necroses 1 cm from left, right and upper border of active growing callus tissue for each tree. After 90 days, the growth (in mm) of the alive tissue toward the nails were measured.

Statistical analysis were processed in R cran (R Core Team, 2013): normality and homogeneity of variance across groups were checked with Shapiro–Wilk Normality Test and Levene, respectively, then Anova ($p < 0.05$) was used to evaluate possible differences. In the case of non normality of data, Kruskall-Wallis test was employed ($p < 0.05$).

Measurement of growing necroses after potassium phosphite treatment

Endotherapic trials were conducted in a chestnut coppice in the Veneto region (Northeastern Italy, coord, 45° 54 ′ 41 ″ N; 12° 2 ′ 10 ″ E; 610 – 630 m a.s.l., approximately 20.3 km airline distant from the site previously described). After careful selection 50 asymptomatic trees were chosen for the experiment, ranging from 7.5 to 14.5 cm (ave. 9.9 cm) diam. at breast height (dbh). In June 2014, controlled artificial inoculations were performed using *P. cinnamomi* strain isolated as previously described and grown on PDA for 7 days at 24 ± 1 °C in the dark. Every trunk was wounded 150 cm above the collar with a sterile 7 mm diam. cork borer, penetrating approximately 5 mm, and a plug of the same diameter removed from the colony edge was placed top side inward into the hole, then protected with the bark previously removed. After 20 days, the edges of the infected wounds were carefully debarked and photographed with a scale bar. Pictures were treated with the computational process as follows. The border of necroses were accurately marked in GIMP v. 2.8 (ANNEX 9; The GIMP team, 2014) and a script in MATLAB v. 8.3 (ANNEX 10; MATLAB, 2014) was created for unwrapping of cylindrical trunk photos, then areas of necroses were measured by means of ImageJ software (v. 1.46r, Wajne Rasband, National Institutes of Health, USA; Abràmoff et al., 2004).

According to both tree diameter and the necrotic areas, the 50 trees were organized into five comparable groups (Peterson et al., 2009), to be injected with commercial formulations of potassium phosphite 35 % as it is (122.5 g/L H_3PO_3; Strazzabosco and Klaudatos, 2013) or in double concentration (70 %; 245 g/L

H_3PO_3; Franceschini, 2011), or with the addition of 20 % allicin (1 g/L) or 0.1 % micronutrient solution (Table 15), and water as a control. Trees were injected at the opposite side 60-70 cm above the inoculation point, in 2-3 equidistant points with 1 mL/cm dbh of each solution. To avoid the production of drill holes, a hollow bladed, manual injection tool was used (BITE, University of Padova pat. n. WO2013010909-A1; Montecchio, 2013; ANNEX 9).

Commercial product	**Active ingredient**	**Strength**	**Manufacturer**
FOSFISAN	P_4O_{10}	30 %	Agrofill by Adriatica S.p.a.
	K_2O	20 %	
CONQUER	Allicin	0.5 %	JCA Limited
AGROVIT L	Soluble B	0.2 %	Agrofill by Adriatica S.p.a.
	Soluble Cu	0.5 %	
	Soluble Fe	0.4 %	
	Fe EDTA	0.4 %	
	Soluble Mn	1 %	
	Soluble Mo	0.02 %	
	Soluble Zn	1 %	

Table 15. Description of the commercial products used in the endotherapic trial.

Treatments effectiveness was assessed by comparing the dimension of the necrotic area measured on the day of the treatment with those observed after 50 days. To verify the presence and vitality of the fungus, two equidistant 3 mm^3 wood samples were collected along the edge of each inoculation point, plated on PDA and incubated for 7 days at 24 ± 1°C in the dark. Isolations were scored as positive when fungal cultures exhibited the typical *P. cinnamomi* morphology (Erwin and Ribeiro, 1996).

Statistical analysis were processed in R cran (R Core Team, 2013). Normality and homogeneity of variance across groups were checked with Shapiro–Wilk

Normality Test and Levene Test ($p > 0.01$ and $p > 0.05$), respectively, then Anova ($p < 0.05$) and Multiple Comparison (TukeyHSD, $p<0.05$) were used to evaluate possible differences on relative ratios of necrotic areas, computed for every tree for last survey date in comparison with the initial one.

Results

Measurement of callus growth after potassium phosphite treatment

The lateral flow test confirmed the presence of *Phytophthora* in the samples from each symptomatic chestnuts. From baiting essay, a *Phytophthora*-like strain was obtained. After treatment with salts, it produced sporangia (average dimensions 75.3 x 47.5 µm, range 43.2-124 x 34.4-72 µm, average length-breast ratio 1.54) and chlamydospores (average diameter 30 µm, range 23.5 - 41.6 µm). According to the white coralloid-type mycelium with abundant hyphal swellings and the measures of fructifications, it could be identified at species level as *P. cinnamomi,* as confirmed by molecular analysis (best match sequence, *Phytophthora cinnamomi*; bit-score, 1130; E value, 0; similarity, 100 %; accession number, KC609419.1; Figure 22).

Considering the endotherapic trial, after 90 days the growth of the callus tissue (Figure 23 for some examples) was not significantly different between the trees treated with potassium phosphite 70 % and water controls (Shapiro–Wilk Normality Test, $p < 0.01$; Levene Test, $p > 0.05$; Kruskall-Wallis Test, $p > 0.05$). Further surveys were not possible because of the fall of some chestnuts included in the trial.

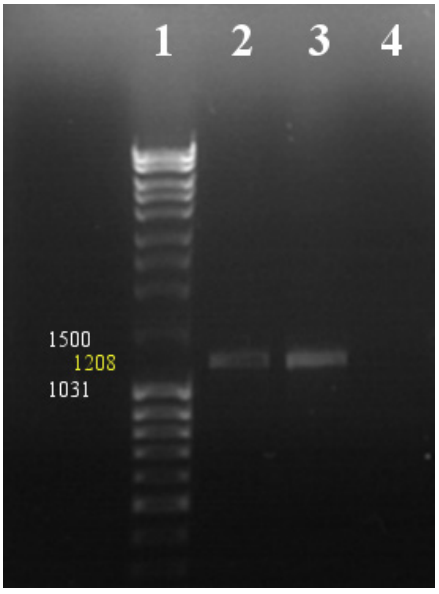

Figure 22. Agarose gel of PCR samples after electrophoresis. Nucleic acids of each sample were loaded on the gel. PCR products are as follows:
lane 1, marker;
lane 2, *P. cinnamomi* isolated with baiting (1/10 dilution);
lane 3, *P. cinnamomi* CBS 144.22 baiting (1/10 dilution);
lane 4, negative control.

Figure 23. Some examples of the possible growth of callus tissue. The initial phase is reported on the left (the day of the treatment), the second phase (90 days after the treatments) on the left. Upper pictures are taken from a control tree, inferior ones represent the border of the necroses of a potassium phosphite treated chestnut (pictures by Dal Maso E.).

Measurement of growing necroses after potassium phosphite treatment

All the inoculation points on chosen chestnuts showed the presence of visibly developed necrotic areas, with great variance in shape and size independent of tree diameter (ANNEX 9).

In the next observation (50 days after the treatments), all the cankers were wider than during injections and none of the products completely blocked the growth of *P. cinnamomi*. Moreover, the pathogen was successfully reisolated from the necroses' edges.

When compared with water injection, all the phosphite treatments slowed down the necroses development accordingly to average values. In particular, potassium phosphite 35 % reduced the growth of the necrosis by 65.5 %, potassium phosphite 70 % by 62.07 %, potassium phosphite plus allicin by 49.2 % and potassium phosphite plus micronutrient by 84.98 % in average (Anova, $p < 0.01$; Shapiro-Wilk Normality Test $p=0.038$; Levene test $p=0.173$; Figure 24). Nevertheless, Multiple Comparison analysis indicated that the growth of the necroses was significantly slower down only by potassium phosphite plus micronutrient solution when compared with water-treated trees ($p<0.05$; Table 16).

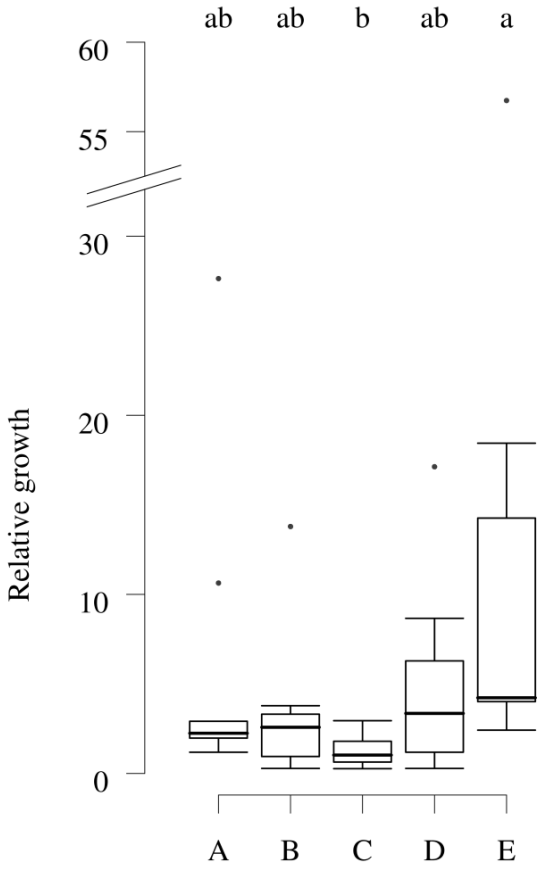

Figure 24. Differences in the relative increase of the necrotic areas after 50 days from the treatments. A = Potassium phosphite 35 %; B = Potassium phosphite 70 %; C = Potassium phosphite 35 % plus micronutrient solution 0.1 %; D = Potassium phosphite 35 % plus allicin solution 20 %; E = Control.

Treatments comparison		**Estimated difference**	**Standard error**	**p value**
Potassium phosphite 35 %	- Control	-7.93	4.173	0.33
Potassium phosphite 70 %	- Control	-10.57	4.067	0.09
Potassium phosphite 35 % *plus* micronutrient solution 0.1 %	- Control	-12.46	4.066	0.03 *
Potassium phosphite 35 % *plus* allicin solution 20 %	- Control	-9.09	4.067	0.19

Table 16. Multiple comparisons (Tukey HSD) between the relative growth of the fungus in the wood in 50 days during the growing season of the treated trees toward water control.

Discussion

New outbreaks of chestnut ink disease (CID), caused by *Phytophthora cinnamomi* and *P. cambivora* (Vannini and Vettraino, 2011; Robin et al., 2012) are increasingly detected in many European chestnut forests and plantations (Turchetti and Maresi, 2008; Beccaro et al., 2009; Costa et al., 2011), reconfirming the interest in management's possibilities. The use of potassium phosphite has become a common practice in the control of many important *Phytophthora* plant diseases, considering also the extremely low toxicity to invertebrates, aquatic organisms and animals (Garbelotto et al., 2007; Thao and Yamakawa, 2009). In particular, against CID, endotherapic treatments were suggested as an effective and environment-friendly method while remaining relatively inexpensive (Tamietti and Valentino, 2005; Gentile et al., 2009; Franceschini, 2011).

In the first endotherapic trial, the injection of potassium phosphite 70 % was evaluated considering growth stimulation of active growing callus next to the shape flame necroses, but the results did not highlight a significant difference between treated trees and water control ones. The outcome could be due to the need of additional time for the evaluation of treatment's effectiveness, but the fall of some chestnuts involved in the study prevented further surveys.

The second study focused on trunk injections of different potassium phosphite formulations against CID. Considering that phosphonates are among the few substances with phloem mobility in the symplast (Ouimette and Coffey, 1990; Brudenell et al., 1995; Garbelotto et al., 2007) and to simulate what could happen treating naturally infected chestnuts, injections were carefully made above the inoculation point at the opposite side. The solution of potassium phosphite at low concentration (35 %) reduced the growth of the necrosis by 65.5 % in average and did not differed significantly from the control. In its comparison, the treatment at double concentration slightly changed the result. This outcome is partly in contrast with those obtained from Tamietti and Valentino (2005); in that trial curative treatment with potassium phosphite on 3 years old trees blocked growing necroses in great part of treated plants after the same interval of time. This differences could be due, for instance, to the different concentration used, the trees' diameters or the pathogenicity of the fungal strain used. Effectively, also Tamietti and Valentino (2005) and Gentile et al. (2009) found difficulty in promoting plant

recovery of older chestnuts heavily affected by CID in the field after potassium phosphite treatment.

Although the antibacterial principle of garlic (*Allium sativum* L.; Slusarenko et al., 2008), allicin, was never directly tested against *P. cinnamomi*, it resulted effective as inhibitor of growth of *P. infestans*, *P. ramorum*, *P. kernoviae*, *P. lateralis* mycelium also at low concentration (Ke-Qiang and van Bruggen, 2001; Curtis et al., 2004; Portz et al., 2008; Hearst et al., 2013). In the current study, *in planta* test on allicin against *P. cinnamomi* did not indicate a significant effect on pathogen growth in comparison to water control; moreover, considering the average reduction of necroses' growth, the addition of allicin to the base solution of potassium phosphite diminished its effective. It is not known whether allicin flows inside the trunk through ascendant xylematic or descendant phloematic streams or both; in the case of main upwards translocation, it would not be able to act against *P. cinnamomi* development below; moreover, it could have combined to the potassium phosphite and carried it upwards.

Best results were achieved by potassium phosphite added with the micronutrient solution, with average 84.98 % necroses development reduction in comparison to the control. Also in this case, the phenomenon could be explained in different ways. As a first hypothesis, direct effect of single component should be considered. Among the elements in the micronutrients solution, the efficacy of soluble copper compounds in the control of *P. cinnamomi* is recognized for a long time (Halsall, 1977; Keast et al., 1985; Coelho et al., 2005). Molybdenum and ferric ions are known to reduce the production of *P. cinnamomi* sporangia *in vitro,* too (Halsall, 1977; Halsall and Forrester, 1977). Moreover, micronutrient solution and phosphonates could act in a synergistic effect; this agrees with the observation made by Darvas et al. (1984) and Bezuidenhout et al. (1987) on phosphate supplemented with zinc sulphate applied to avocado affected by *P. cinnamomi.* Micronutrient injection could also have influenced the defense response to the pathogen, as systemic protection could be attributed to the nutrients increasing plant cell resistance (Reuveni et al., 1997; Simoglou and Dordas, 2006; Frenkel et al., 2010). At last, the destruction of the root system of chestnuts infected by *P. cinnamomi* in infested groves reduces nutrient uptake and limit tree's growth (Labanauskas et al., 1976; Portela et al., 1999; Maurel et al., 2001). Therefore, injection of low concentration micronutrients, such as manganese and iron

necessary for photosynthesis (Puig and Peñarrubia, 2009; Thomine and Vert, 2013), could provide for this deficiencies.

The results of this study implement the knowledge base on CID endotherapic treatments with potassium phosphite, indicating that the addition of micronutrient solution can significantly slow down the development of the disease for at least few months. Moreover, endotherapic treatments, delivering agents directly into trees, well adapt to a general context with people more concerned about the effects of pesticides on humans and the environment generally (Ferracini and Alma, 2008; Tanis et al., 2012). Further investigations will assess the efficacy of various formulations in terms of different chestnuts age classes and genotypes, from a preventive and curative view.

CHAPTER VI

General discussion and conclusions

The results presented in this thesis regard the development of updated and innovative methods in the management of forest diseases. From a perspective of plant disease management, practices rely on anticipating occurrence of disease (prevention; i.e monitoring environmental factors for disease forecasting, site selection and preparation, utilizing resistant cultivars, altering planting practices, drainage, irrigation, pruning, thinning, shading) and therapy (treatment or cure; Maloy, 2005). In this context, environmental modelling for risk prediction and endotherapic treatments were tested on two major forest diseases, ash dieback caused by *Hymenoscyphus fraxineus* and chestnut ink disease (CID) caused by *Phytophthora cinnamomi* Rands and *Phytophthora cambivora* (Petri) Buism. The two diseases are extremely different, both for epidemiology, pathogenesis, hosts and knowledge base. The rapid spread of the airborne *H. fraxineus* in Europe was reported only in 2006 (Kowalski, 2006; EPPO, 2013a; Gross et al., 2014a). In contrast, CID is known since 1917 (Petri, 1917), but new outbreaks are increasingly detected in many European chestnut forests and plantations (Turchetti and Maresi, 2008; Vettraino et al., 2008; Beccaro et al., 2009; Costa et al., 2011; Woodward et al., 2011), reconfirming the interest in management's possibilities.

Predicting the spread of infectious diseases is fundamental for forecasting potential ecological consequences and designing control strategies, in a risk assessment context (Brown et al. 2005; Sansford et al. 2009; Dupin et al., 2011; Meentemeyer et al., 2011; Robinet et al. 2012; Santini et al., 2013), and mathematical models have long been widely used for agricultural and forest diseases (Van Maanen and Xu, 2003; Bergot et al., 2004; Meentemeyer et al., 2004; Venette and Cohen, 2006; Kelly et al., 2007; Ganley et al., 2009; Klopfenstein et al., 2009). Among those extensively employed, habitat suitability models (environmental niche models) can be constructed utilizing spatial analysis methods, which relate the presence or absence of the target species to a set of environmental variables (Iverson and Prasad, 1998; Kelly et al., 2007; Kamino et al., 2012). In the last years the variety of techniques used for ecological modelling has increased (Guisan and Zimmermann, 2000; ANNEX 2). In order to assess the infection risk in pest-free areas by *H. fraxineus*, the *consensus ensemble forecast* technique developed in this thesis permitted to highlight the suitable areas at European scale within European ash species ranges. Moreover, it lead to interesting conclusions on the ecological appropriateness of some areas to the potential pathogen spread,

considering the principal environmental features that characterize naturally infected zones. In particular, pathogen's natural presence was highly correlated with abundant rainfall, high soil moisture content and low mean temperatures in the summer months. Furthermore, a network analysis permitted dispersal dynamics to be included in order to obtain realistic risk predictions for natural spread. As a general consideration, this type of models, which can increase ecological understanding for cases where the knowledge is relative low (Wainwright and Mullligan, 2013), represents a promising tool in the spatial prediction of plant pathogen species for which environmental requirements are still little investigated. As disadvantages, these mathematical models usually have low interpretability (the so called "black-box", Elith and Leathwick, 2009) and possibly require a continuous update, for instance, with wider time series, data on disease severity and hosts abundance, to potentially enlarge the boundaries of the Grinellian niche (closer to equilibrium, according to Pulliam, 2000; Václavík and Meentemeyer, 2012).

In the case of CID, a completely different approach was implemented and the fuzzy-rule based theory was used to estimate habitat suitability at large scale in the Treviso province. Fuzzy set theory offers good predictive capability and reasonable estimates of the unknown model parameters inherent to variables and functions of complex ecosystems (Omlin and Reichert, 1999; Adrianssens et al., 2004; Fukuda, 2009). Fuzzy logic, widely used in engineering and process control sciences (Sugeno, 1985; Von Altrock, 1995), was then applied in biology end environmental sciences (Ayyub and McCuen, 1987; Equihua, 1990). In plant pathology, it found first applications in disease intensity prediction (i.e. coffee and soybean rust; Kim et al., 2005; Alves et al., 2011), infection simulation (i.e. grapevine downy mildew; Orlandini et al., 2003) and diagnosis (i.e. oilseeds crops; Kolhe et al., 2011), and risk assessment (i.e. European canker of apple; Kim and Beresford, 2012). In this thesis, the fuzzy model created for CID, here employed in forest pathology for the first time, permitted the ranking of a current or potential chestnut area for probabilities of CID development, considering the environmental variables associated to host presence and parasites' ecological niches. The effectiveness of the rule-based modelling outcomes, supplied by uncertainty maps for their correct interpretation, was confirmed by detailed field data collection. In comparison to the model applied to ash dieback, the use of fuzzy model technique did not require a training dataset, basing directly in *a priori* understanding of the ecological

characteristics of CID. Fuzzy models demonstrated to be simple (relations between input and output are explained with linguistic rules) and robust (performance is not depending on the training data; Adrianssens et al., 2004, 2006). Therefore, for well known pathogens, this methodology can allow resources' saving, which can be devolved in specific validation data collection. Undoubtedly, data-driven technique can be employed in fuzzy logic too, by many authors recommended to provide a preliminary model to the experts (Mouton et al., 2011), but this approach is extremely data hungry and has very limited transportability (Zhu et al., 2014).

The two bioclimatic modelling techniques exhibit also different approaches towards the fundamental uncertainty factor. The consensus modelling framework (ensemble forecasting) procedure can enable robust decision making in the face of uncertainty, in particular in a conservation planning context (Araújo and New, 2007), because it tones down the errors of the single models. On the contrary, the fuzzy model directly incorporates uncertainty in model construction (i.e. in the size of membership functions overlapping) and it permits also to consider the weights of every key source of uncertainty, in particular model's structure, input data and parameter values, on the final prediction (Refsgaard et al., 2006). For instance, in the CID application of fuzzy logic in this thesis, it was possible to notice that underpredictions (false negative observations) detected in the validation procedure were included in transitional areas with the highest input uncertainty.

The optimal model should provide the greatest simplifications together with an accurate representation of the investigated phenomenon. Currently, there is no way to assess which niche-based model is the most appropriate in forest pathogens' epidemiology prediction (Thuiller, 2004). Considering the reported results, the choice should be made on the basis of the availability of biological and ecological information, explanatory variables at the study scale and the possibility to collect survey data for model construction and validation. Once built the chosen model, the possibility of updating with different environmental predictors or biological information, should be highly regarded.

The second principle of plant disease management, the therapy, was deepened with endotherapic treatments trials, with successful results in both cases, ash dieback and CID. Endotherapy has been long applied in tree care as a preventive and curative treatment, with first trials by Leonardo Da Vinci in the 15th century (Da Vinci, 1478-1519a,b; Tattar et al., 1998; Sánchez-Zamora and Fernández-

Escobar, 2004; Ferracini and Alma, 2008; Montecchio, 2013; Aćimović et al., 2014); nevertheless, in the case of new plant pathogen species information are naturally lacking (Hubbard and Potter, 2006), in the case of old known disease, data are often localized or incomplete for a broad use of this technique. In the present thesis, trunk injections were applied against the two above mentioned diseases, in order to supply useful information on the efficient use of trunk injection.

In the case of ash dieback, considering the partly knowledge of effective compounds against the pathogen (Hauptman et al., 2012; Cooke et al., 2013), *in vitro* tests were firstly performed and results indicated thiabendazole, propiconazole and allicin as the best performing agents. Subsequently, their antifungal activities were investigated *in planta* against *H. fraxineus* by trunk injection. *Fraxinus* genus was indicated as difficult to inject in the past (Sach et al., 1986), so *in planta* tests were preceded by preliminary trials that indicated higher treatment velocity when performing trunk injections at breast height in early morning or late afternoon with the addition of a small amount (1.2 %) of acetic acid. The final efficacy tests of fungicides *in planta* indicated allicin and thiabendazole as effective in slowing down the pathogen growth and acceptable efficient in injectability.

Taking into account CID complex, the efficacy of phosphonates against *P. cinnamomi* and *P. cambivora in vitro* is known for a long time (Coelho et al., 2005; Hardy et al., 2001; Wilkinson et al., 2001; Gouveia et al., 2010) and endotherapic treatments were suggested as an effective and environment-friendly method while remaining relatively inexpensive (Tamietti and Valentino, 2005; Gentile et al., 2009; Franceschini, 2011), but little was known about ideal concentration and potential adjuvants. The study conducted in this thesis permitted to evaluate different potassium phosphite formulations against CID *in planta* in a comparative trial. Trunk injection was performed on fifty asymptomatic sweet chestnuts inoculated with *P. cinnamomi*, isolated with baiting technique from diseased chestnuts in a near zone. Although pure solutions of potassium phosphite (35 % and 70 %) reduced the growth of *P. cinnamomi* in average, the only formulation that significantly slowed down the necroses consisted in potassium phosphite added with a micronutrient solution. On the contrary, the addition of allicin to the phosphite solution reduced its effective. In another trial, the main aim was to ascertain the possible growth of the active callus on necroses border after potassium phosphite injection, but the treatment did not give different results in comparison

to water control, probably for longer times need for efficacy evidence.

Endotherapic treatments, delivering agents directly into trees, well adapt to a general context with people more concerned about the effects of pesticides on humans and the environment and restricted possibility of fungicide use (Ferracini and Alma, 2008; European Commission, 2009; Tanis et al., 2012; Wise et al., 2014). Indeed, they represent an environmentally safer alternative to the traditional approach of spraying chemicals, in line with the current legislation on the use of fungicides (i.e. Directive 2009/128/EC at European level; Pavela and Bárnet, 2005; Montecchio, 2013). From this perspective, biological control acquires increasingly importance (Santamaría et al., 2007). As limitations, trunk injection technique requires a specialized knowledge not only on the practical aspects on the used tool and best formulations, but also on the ecophysiology, phenology and phytosanitary status on the tree of interest. Nevertheless, while a blanket use would probably be impractical and expensive, endotherapy could recover special historic and high value trees (Marshall, 2014) and further studies are essential to create technical execution tables for each species, host genotype, disease, stand conditions combinations, in order to make treatment ever more safe, effective and efficient.

As general conclusions, risk predictions maps obtained by means of bioclimatic environment niche modelling, represent an useful and cost-effective decision support tool in Pest Risk Management, linking their outcomes to allow better forest conservation strategies, aid monitoring survey, focus prompter phytosanitary measures along an outbreak, and promote discussions about the control of the disease and the risks associated to trade or movement of plants for plantings in the perspective of global economy. From a curative point of view, endotherapic treatments represent an useful tool both effective, because active ingredients are delivered directly to the target pest for optimal exposure, and environmental safe, because it eliminates or significantly reduces off-target drift-driven pesticide losses to the environment. These preventive and curative measures should be applied in a coordinated integrated and harmonized manner, together with the appropriate cultural practices, to maximize benefits.

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LUCHI N., MONTECCHIO L., DAL MASO E., SANTINI A., 2012 Situation with Ash in Italy: stand characteristics, health condition, ongoing work and research needs. 1st FRAXBACK WG/MC Meeting, 13-14 Novembre 2012, Vilnius, Lituania.

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DAL MASO E., MONTECCHIO L., 2014. Comparative trials of four potassium phosphite formulations against chestnut ink disease by trunk injection. VII Congress on Plant Protection, "Integrated plant protection knowledge - based step toward sustainable agriculture, forestry and landscape architecture". November 24-28, 2014, Zlatibor, Serbia. Book of Abstracts, ISBN: 978-86-83017-25-6, Belgrade: Kaktusprint. pp. 94-95.

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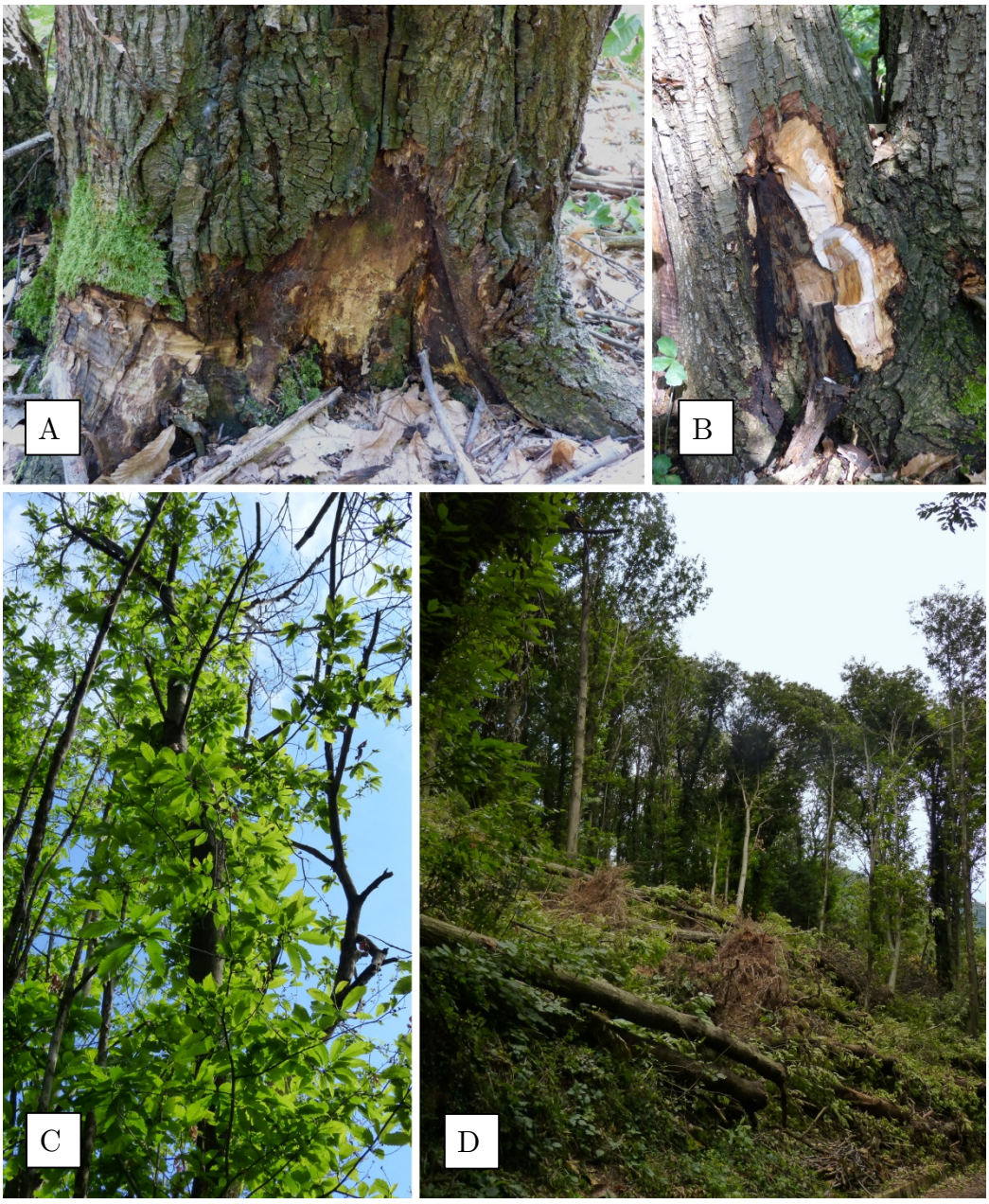
ANNEX 1

Chestnut ink disease symptoms and compromised slope



A. Shape flame necroses at trunk collar on a symptomatic chestnut and upper cracked area; B. Debarked necroses and callus in order to show the sharp transition between the necrotic and the healthy areas; C. Symptomatic chestnut with canopy dieback; D. A compromised slope, with crashed chestnuts affected by *Phytophthora* spp. (pictures by Dal Maso E.).

ANNEX 2

Epidemiological forecasting modelling - An overview

Epidemic derives from two Greek words, *epi* (on/among) and *demon* (population), meaning "any phenomenon affecting most of the individuals of a population in progress at the same place and at the same time". Indeed, this term assumes a change in disease intensity in a host population over time and space. The world *epiphytotic* or botanical epidemiology has been used in scientific literature, but was abandoned with the increased common use of "epidemiology" word (Chaube and Pundhir, 2005).

Elements of epidemics

Plant disease epidemic can develop as a result of a combination of three main factors, the classical disease triangle, comprising a susceptible host, a virulent pathogen and favorable environmental conditions (Agrios, 2005). Host population is characterized by the degree of genetic uniformity, age, vertical or horizontal resistance and crop duration (annual or perennial). Furthermore, the pathogen

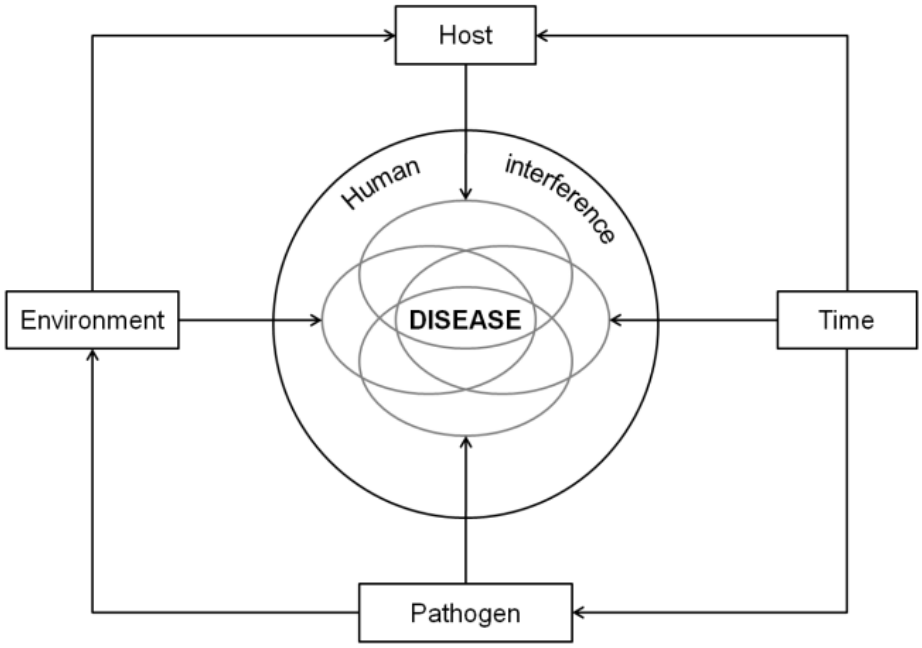


Figure 25. Schematic representation of the elements of an epidemic (Chaube and Pundhir, 2005, modified).

mycelium, spores, cleistothecia) and quantity of inoculum near hosts, the type of factor can present differences on the base of the level of virulence, the type (i.e. reproduction (monocyclic or polycyclic), latent and infectious periods and the mode of spread (i.e. air-borne, soil-borne or seed-borne; Cooke et al., 2006). The main environmental features involved in disease development are moisture (i.e. rain), temperature (Agrios, 2005), radiation and wind speed (Contreras-Medina et al., 2009). Moreover, the triangle can be developed into a tetrahedon (Francl, 2007) or a pyramid, adding humans and time as additional two components (Figure 25). In particular, humans can chose the kind, the number and the density of plant grown, the cultural practices, the chemical or organic control; moreover humans can be responsible of the introduction of new pathogens. On the other hand, disease should be plotted against time factor, comprising, i.e., the season and the duration of favorable meteorological conditions (Agrios, 2005).

Epidemiological modelling and disease forecasting

Epidemiological models aim to interpret the biology of the pathogen in the context of conditions which affect its development, survival and ability to infect and colonize the relevant host, in order to develop sustainable strategies for disease management (van Maanen and Xu, 2003). Unfortunately, it is unlikely that all eventualities will be covered by even the most complex of models because biological processes are in a constant state of flux (i.e. with the introduction of new strains and mating types, rapid changes in aggressiveness and temperature adaptations; Cooke et al., 2006). For this reason, most of the models impose a cut-off and the degree of precision and complexity of the required modelling, and analysis is determined by the question(s) modelers are trying to answer. In plant pathology, the main objective for modelling is the prediction of a probable outbreak or increases in intensity of disease, for the formulation of control measures (Agrios, 2005). In particular, Norton and Mumford (1993) and van Maanen and Xu (2003) proposed this classification of objectives:

- Predicting the time of an event;
- Predicting the scale of an event;
- Estimating the frequency or probability of an event (i.e. in polycyclic epidemics);
- Comparing the performance of different management strategies.

Disease forecasting is therefore required both for economic and safety reasons, because it can reduce the cost of production (i.e. rational treatments) and the effects of pesticides toward the environment, operators and consumers (Cooke et al., 2006). According to Campbell and Madden (1990) and Esker et al. (2008), the success of plant disease forecasting system should be evaluated on reliability (use of sound biological and environmental data), simplicity, importance (i.e. economic reply) and usefulness, availability of information about the components, multipurpose applicability for several diseases and pests and, finally, cost effectiveness.

The most commons mathematical tools to describe epidemic dynamics can be summarized as follows:

- Disease progress curve (growth model)

This solution permits to analyze individual epidemics considering the temporal dimension. The pattern of the epidemics is quantified in the terms of number of lesions, amount of diseased tissue or incidence (Agrios, 2005; Hau and Kosman, 2007) and plotted over time with a disease-progress curve. The growth models commonly used are the monomolecular, exponential (or logarithmic or Malthusian), Gompertz and logistic, all in differential form (Figure 26). The time independent variable can be the calendar time (days, weeks), or, as suggested in the case of epidemics comparisons, the "biological time" (Cooke et al., 2006). In particular, monomolecular model is appropriate when the pathogen has a single cycle during the growing season; the exponential curve can describe the very early stages of most polycyclic epidemics; the widely used logistic and the alternative Gompertz model are indicated for polycyclic epidemics.

Generally, these models have been used to describe the observed patterns and compare epidemics. As main limitation, this kind of models are simple and can ignore several important factors such as host growth and fluctuating environmental conditions, with direct or indirect effect on disease development (Cooke et al., 2006).

Examples. There are many publications comprising the use of the growth curves in plant pathology. In particular, exponential curves were used, for instance, in the quantification of disease expression at different stages (Daamen, 1991; de Jong,

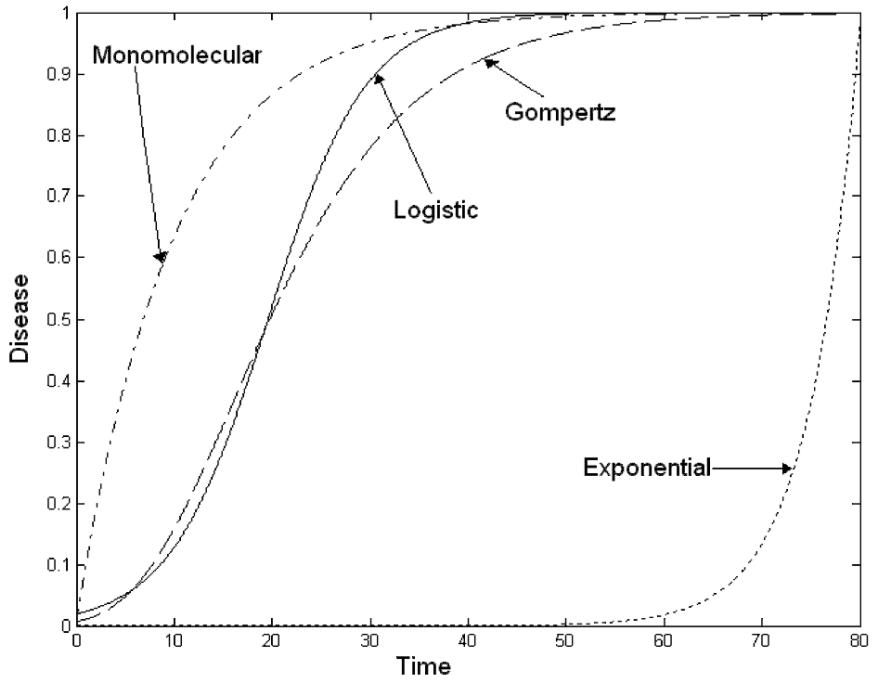


Figure 26. Examples of disease progress curves (Contreras-Medina et al., 2009, modified).

1995; Cao et al., 2012) or sometimes integrated with weather variables (Papastamati and van den Bosch, 2007), or, rarely, as a dispersal function over space (Wingen et al., 2013). Monomolecular model are usually used to describe the progress of a disease over time (Kravchenko et al., 2011; Gongora-Canul et al., 2012; Vicent et al., 2012; Hughes et al., 2014). The Gompertz and the logistic equation were used originally to fit the growth of bacteria and to determine the time of growth and toxin production by *Clostridium botulinum*, respectively (Dantigny et al., 2011). Later, the models were usually test together, to fit the germination data (Marín et al., 1996; Dantigny et al., 2002; Huang et al., 2010), disease progress over time (Café-Filho et al., 2010; Bernard et al., 2013; Hughes et al., 2014) or disease expression and infection pressure (Jarroudi et al., 2012), although logistic model has more applications (Paul and Munkvold, 2004; Henderson et al., 2007; Harikrishnan and del Río, 2008; Guyader et al., 2013; Shah et al., 2013; Xu et al., 2013; McKay et al., 2014). However, great part of papers compare the performances of more than one growth curve and chose the best one on the base of the highest Goodness of Fit (Scott et al., 2003; Beltrán et al., 2008; Batzer et al., 2012; Carisse et al., 2014).

- Area under the disease progress curve

The area under the disease progress curve (AUDPC) is a quantitative summary of disease intensity over time, for comparison across years, locations, or management tactics. The most commonly used method for estimating the AUDPC is the trapezoidal method. It permits to discretize the time variable and calculate the average disease intensity or economic loss between each pair of adjacent time points, without regard to curve shape (Figure 27 for an example; Sparks et al., 2008). AUDPC can be very useful as an alternative to fitting growth models when observed disease patterns cannot be fitted to a progress curve (Cooke et al., 2006).

Examples. AUDPC computation is usually used to assess different resistance to disease (van Maanen and Xu, 2003; Lecomte et al., 2014; Mirkarimi et al., 2013; Paraschivu et al., 2013), the virulence of pathogens (Purahong et al., 2014; Shishido et al., 2014), effectiveness of various treatments against the disease (Conceição et al., 2014; Senechkin et al., 2014) and pathogenesis comparing different host parts or time of infection (Siou et al., 2014).

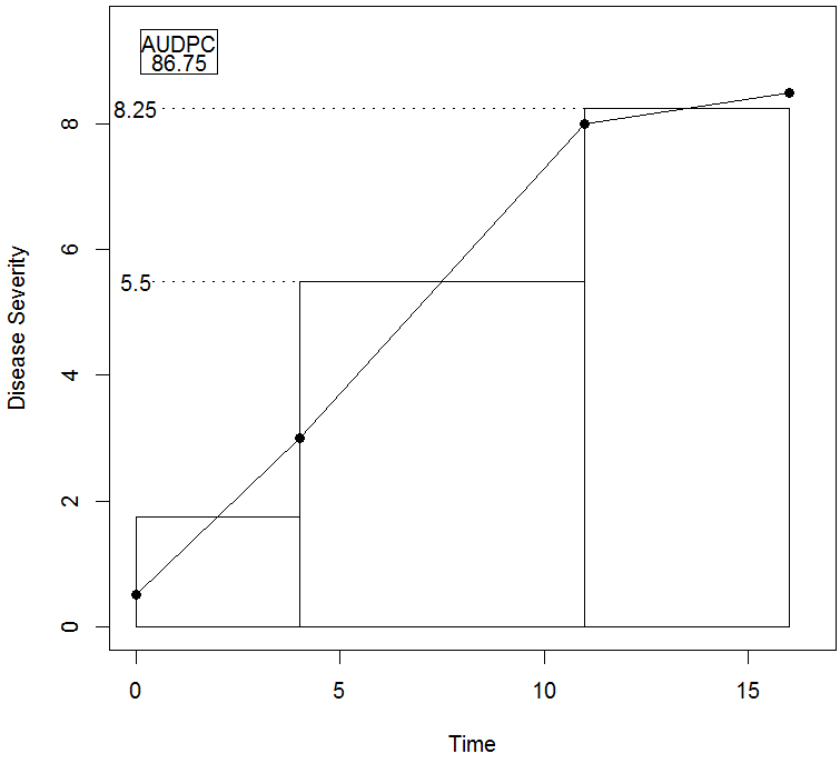


Figure 27. Example of AUDPC computation, conducted in R cran.

- Linked differential equations and **computer simulation**

Linked differential equations (LDE) represent a flexible and biologically intuitive approach to incorporate many disease components into a single model (Cooke et al., 2006). In this type of model, differential equations aim to interpret the dynamics of plant disease in relation to host, environment and human interventions. The LDE models are of the susceptible (healthy), infected and removed (post-infectious) (SIR) type or of the susceptible, exposed, infected and removed (SEIR) type, usually used in human diseases' epidemiology (Van Maanen and Xu, 2003; Scherm et al., 2006).

Examples. Some examples of the use of LDE and computer simulation are reported in Table 17.

Model name	**Host(s)**	**Pathogen**	**Reference**
Lalancette et al.	Grapevine	*Plasmopara viticola*	Lalancette et al., 1988
MILVIT	Grapevine	*Plasmopara viticola*	Magnien et al., 1991
PLASMO	Grapevine	*Plasmopara viticola*	Rosa et al., 1993, Orlandini et al., 1993
Rossi et al	Grapevine	*Plasmopara viticola*	Rossi et al., 2007a, Rossi et al., 2009
VITIMETEO	Grapevine	*Plasmopara viticola*	Bleyer et al., 2008, Viret et al., 2005
Orlandini et al.	Grapevine	*Plasmopara viticola*	Orlandini et al., 2008
Thomas et al.	Grapevine	*Uncinula necator*	Thomas et al., 1994
Nair et al.	Grapevine	*Botrytis cinerea*	Nair and Allen, 1993
Xu et al.	Apple	*Venturia inaequalis*	Xu et al., 1995
RIMPRO	Apple	*Venturia inaequalis*	Trapman and Polfiet, 1997
A-Scab	Apple	*Venturia inaequalis*	Rossi et al., 2007b
Rossi et al.	Apple	*Venturia inaequalis*	Rossi et al., 2003a
Adem	Apple	*Venturia inaequalis*	Berrie and Xu, 2003
VENTEM	Apple	*Venturia inaequalis*	Van Santen and Butt, 1992
DLV-Welte and RIMpro	Apple, Pear	*Venturia inaequalis* and *Venturia pirina*	Aalber et al., 2001
BSP-Cast	Pear	*Stemphylium vesicarium*	Montesinos et al., 1995, Llorente et al., 2000
Cougarblight	Apple, Pear	*Erwinia amylovora*	Dewdney et al., 2007, Smith and Pusey, 2011
MARYBLYT	Pear	*Erwinia amylovora*	Steiner, 1990
Smith	Pear	*Erwinia amylovora*	Smith, 1993
Bugiani et al	Tomato	*Phytophthora infestans*	Bugiani et al., 1993

PhytoPRE	Tomato	*Phytophthora infestans*	Forrer et al., 1993
PROGEB	Tomato	*Phytophthora infestans*	Gutsche, 1993
BLITECAST	Potato	*Phytophthora infestans*	Krause et al., 1975
De Visser	Onion	*Botryotinia squamosa, Peronospora destructor*	De Visser, 1996
ONIMIL	Onion	*Peronospora destructor*	Battilani et al., 1996
MILIONCAST	Onion	*Peronospora destructor*	Gilles et al., 2004
CERCOPRI	Sugarbeet	*Cercospora beticola*	Rossi and Battilani 2008
CERCODEP	Sugarbeet	*Cercospora beticola*	Rossi et al., 1994
CERMAL	Wheat	Various	Battilani et al., 1993
RUSTDEP	Wheat	*Puccinia recondita*	Rossi et al., 1997
SRESM	Wheat	*Puccinia striiformis*	Luo and Zheng, 1995
Rossi et al.	Wheat	*Gibberella zeae*, *Fusarium culmorum*, *Gibberella avenacea*, *Monographella nivalis*	Rossi et al., 2003b
POWPRI	Wheat	*Erysiphe graminis*	Rossi et al., 2000a, Rossi et al., 2000b
Battilani et al.	Sunflower	*Diaporthe helianthi*	Battilani et al., 2003
Aguayo 2014	Alder	*Phytophthora alni*	Aguayo et al., 2014

Table 17. Some examples of the use of LDE in plant pathology.

Steady-state analysis of these equations may generate important results, for example on criteria for persistence and invasion (Cooke et al., 2006), but the purpose of this theory is to explain rather to predict or contribute to the development of new theories (Scherm et al., 2006). Computer simulation have therefore be applied together with LDE in epidemiological modelling. Usually, each subcomponent of disease development (i.e. the stage of the life cycle of the pathogen) is considered in a dynamic LDE approach (Contreras-Medina et al., 2009). Computer simulation may serve not only as an educational platform, but overall to evaluate the importance of each subcomponents of an epidemic at a particular time, in order to indicate the most effective management (Agrios, 2005).

- Statistical tools

In few cases, linear or multiple regression can be used for describing the relationship between disease severity and time (Sparks et al., 2008). On the contrary statistical tools can be applied in some stages of the epidemiological model construction, overall when little is known about the structural form of complex relationships between response variables (Contreras-Medina et al., 2009). Several statistical tools have the potential to improve inference from a range of epidemiological studies, such as generalized linear mixed models (GLMMs), Bayesian analysis and ROC analysis in decision support, genetic algorithms (Scherm et al., 2006), principal component analysis (PCA) and factor analysis (FA) in reducing variables and data dimensions. Other tools can be used in comparing epidemics, such as analysis of variance (ANOVA), residual (restricted) maximum likelihood (REML), cluster analysis, canonical variate analysis, discriminant function/logistic regression, survival analysis and canonical correlation (Cooke et al., 2006).

- Future trends

Nowadays, there are many tools which started to be applied in plant disease epidemiological modelling, overall integrating the pathogen identification phase with greater speed, volume and accuracy, and environmental variables data collection. These tools include:

> Photosynthetic measurement systems
> Molecular Tools
> Geographic Information System (GIS)
> Global Positioning System (GPS)
> Geostatistics
> Remote Sensing
> Multi spectral and continuous spectrum scans
> Digital plant canopy imager
> Image Analysis (e.g. lesion/colony area, space fill)
> Information Technology

(Agrios, 2005, Cooke et al., 2006)

Integration of Species distribution modelling in plant disease epidemiology

Species distribution modelling (SDM; including habitat modelling and ecological niche modelling) refers to statistical and/or mechanistic approaches to the assessment of range determinants and prediction of species occurrence across space and/or time (Svenning et al., 2011). In other words, it quantifies the correlation between environmental factors and the distribution of plant and animal species (Miller, 2010). Predictions from these models inform conservation policy, invasive species management and disease-control measures, focusing on distribution shift of the considered species caused by ecological problems (i.e climate change, habitat fragmentation and biological invasion; Beale and Lennon, 2012). Moreover, they have been used to study the relationships between environmental parameters and species richness, and characteristics and spatial configuration of habitats that allow persistence of species in landscapes and invasive potential of non-native species (Elith et al., 2006). The majority of papers on SDM regard niche-based distribution models. They focus on estimation of a species' niche from the geographical distribution of species (field observations), considering each environmental gradient as a single dimension in Hutchinson's *n*-dimensional niche (Miller, 2010). Therefore, models can be based on a variety of climatic or other environmental variables, for example temperature, precipitation, elevation, ground cover or soil type (Richards et al., 2007). At last, predictions (in space and time) can be made by re-projection to different geographical space (Guisan and Thuiller, 2005; Beale and Lennon, 2012). A huge range of methods have been developed for this theory (Guisan and Thuiller, 2005), from statistical regression methods, such as generalized-linear models and generalized-additive models, to machine-learning approaches, including artificial neural networks and implementations of genetic or other learning algorithms (Beale and Lennon, 2012). Utilization of SDM has grown substantially during the last decade thanks to geographic information systems (GIS) and the rapidly increasing wealth of environmental data (Svenning et al., 2011; Miller, 2010).

Although well established in many fields of biological research, SDMs are still uncommon in plant pathology. There are few examples of application and these regard: the influence of environment and climate on occurrence of the cixiid

planthopper *Hyalesthes obsoletus* (the vector of the grapevine disease 'bois noir'; Panassiti et al., 2013); the prediction of the biological invasion by *Phytophthora ramorum* (Meentemeyer et al., 2008; Václavík and Meentemeyer, 2009; Václavík et al., 2010); the probability of gypsy moth (*Lymantria dispar* L.) establishment (Lippitt, 2008); four key diseases for cassava in developing countries (whiteflies, cassava green mites, cassava mosaic disease and cassava brown streak disease; Campo et al., 2011) and the prediction of the range of the leafhopper, *Hishimonus phycitis*, in Iran (Shabani et al., 2012).

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ANNEX 3

R code for the construction of the spatially explicit model for *H. fraxineus*

```
## Opening useful libraries
library(maptools)
library(rgdal)
library(maps)
library(shapefiles)
library(spdep)
## Reading in data
mapIDs <-
readShapeSpatial("C:/Users/    (insert    here    the    folder
containing the downloaded map) /PresenceMap")
## A quick summary of the data
summary(mapIDs)
attributes(mapIDs)
names(mapIDs)
plot(mapIDs)
class(mapIDs)
str(mapIDs)
## Preliminary settings
coords <- coordinates(mapIDs)
set.ZeroPolicyOption(TRUE)
## Network creation and visualization
Net<- poly2nb(mapIDs, queen = FALSE, snap = 1.45)
isTRUE(all.equal(mapIDs, Net, check.attributes = FALSE))
summary(Net)
plot(mapIDs, col = "grey95", border = "grey")
plot(Net, coordinates(mapIDs), add = TRUE, pch = 16, lwd = 1,
cex = 1, col = "blue")
## Obtaining the matrix of distance
DistanceMatrix <- nb2mat(Net, glist=NULL, style="B")
dim(DistanceMatrix)
ncol(DistanceMatrix)
nrow(DistanceMatrix)
w.cols <- 1:883
w.rows <- 1:883
## Vector presences creation
```

```
vectorIDs<-cbind(mapIDs$PRESABS)
vectorIDs
str(vectorIDs)
## Simulation (x100)  - Attention: running this part could
# require long time and availability of computer memory space
Index = 1:100
for(i in 1:length(Index)) {
for(i in 1:883)
{ for(j in 1:883)
{  if(DistanceMatrix  [[i,j]]  >  0  &  (vectorIDs[[i]]  >  0
|   vectorIDs[[j]]   >   0))   {(vectorIDs[[i]]   <-   1)   &&
(vectorIDs[j] <- 1)
print(vectorIDs)
} } } }
## Transformation of the final vector in dataframe
IDs <- c(1:883)
dataframeIDs<-data.frame(vectorIDs, IDs)
dataframeIDs
## Creation of the final map and control
mapIDsFinal<-SpatialPolygonsDataFrame(mapIDs,dataframeIDs,
match.ID = FALSE)
summary(mapIDsFinal)attributes(mapIDsFinal)
names(mapIDsFinal)
str(mapIDsFinal)
## Export the map
writePolyShape(mappaIDsbindPol,   "C:/Users/   (insert   here
the folder) /FinalMap")
```

ANNEX 4

Principal optimized parameters for the single models

Generalized Linear Model (GLM)

Distribution: Binomial

Link function: Negative binomial

Singularity tolerance: 1.0E-7

Logistic Regression Model (LOG), 1° order interactions

Method: Backward stepwise removal

Singularity tolerance: 1.0E-5

Probability for entry: 0.75

Probability for removal: 0.76

Logistic Regression Model (LOG), main effects

Method: Backward stepwise removal

Singularity tolerance: 1.0E-10

Probability for entry: 0.001

Probability for removal: 0.005

Chi-squared Automatic Interaction Detector Classification Tree (CHAID), boosting

Maximum tree depth: 10

Minimum percentage for parent nodes: 1 %

Minimum percentage for parent for child nodes: 0.5 %

Significance level for splitting nodes: 0.1

Significance level for merging nodes: 0.1

Chi-squared Automatic Interaction Detector Classification Tree (CHAID), bagging

Maximum tree depth: 10

Minimum percentage for parent nodes: 1 %

Minimum percentage for parent for child nodes: 0.5 %
Significance level for splitting nodes: 0.1
Significance level for merging nodes: 0.1

Multilayer Perceptron Artificial Neural Network (MLP), boosting
Minimum precision: 98 %
Overfit prevention criterion: 30 %
Combination rule: median

Multilayer Perceptron Artificial Neural Network (MLP), bagging
Minimum precision: 98.6 %
Overfit prevention criterion: 20 %
Combination rule: average

Support Vector Machine Model (SVM)
Kernel function: RBF
Gamma: 2.5
Regularization parameter (C): 16
Regression precision (epsilon): 5

Maxent
Note: in Maxent software (v 3.3.3k), automatic setting is usually recommended.
Regularization multiplier: 1
Feature type: Auto features
Replicated run type: Crossvalidation
Adjust sample radius: 1

ANNEX 5

Contingency tables for the evaluation of the singles model on the test set

Example of interpretation of a contingency table

		Prediction		
		0	1	Total
Test set	0	TN	FP	TN + FP
	1	FN	TP	FN + TP
Total		TN + FN	FP + TP	*n*

Performances were computed on the test set. Abbreviations: n, total number of cases; TN, true negative; FP, false positive; TP, true positive; FN, false negative; "0", pseudoabsences; "1", presences. Grand total is in italic.

Generalised Linear Model (GLM)

		Prediction		
		0	1	Total
Test set	0	96	19	115
	1	9	22	31
Total		105	41	*146*

Logistic Regression Model (LOG), main effects

		Prediction		
		0	1	Total
Test set	0	101	14	115
	1	13	18	31
Total		114	32	*146*

Logistic Regression Model (LOG), 1° order interactions

		Prediction		
		0	1	Total
Test set	0	60	55	115
	1	11	20	31
Total		71	75	*146*

Support Vector Machine Model (SVM)

		Prediction		
		0	1	Total
Test set	0	105	10	115
	1	6	25	31
Total		111	35	*146*

Multilayer Perceptron Artificial Neural Network (MLP), boosting

		Prediction		
		0	1	Total
Test set	0	108	7	115
	1	8	23	31
Total		116	30	*146*

Multilayer Perceptron Artificial Neural Network (MLP), bagging

		Prediction		
		0	1	Total
Test set	0	101	14	115
	1	9	22	31
Total		110	36	*146*

Chi-squared Automatic Interaction Detector Classification Tree (CHAID), boosting

		Prediction		
		0	1	Total
Test set	0	101	14	115
	1	13	18	31
Total		114	32	*146*

Chi-squared Automatic Interaction Detector Classification Tree (CHAID), bagging

		Prediction		
		0	1	Total
Test set	0	101	14	115
	1	14	17	31
Total		115	31	*146*

Maxent

		Prediction		
		0	1	Total
Test set	0	104	11	115
	1	11	20	31
Total		115	31	*146*

Weighted average (WA) consensus model

		Prediction		
		0	1	Total
Test set	0	107	8	115
	1	7	24	31
Total		114	32	*146*

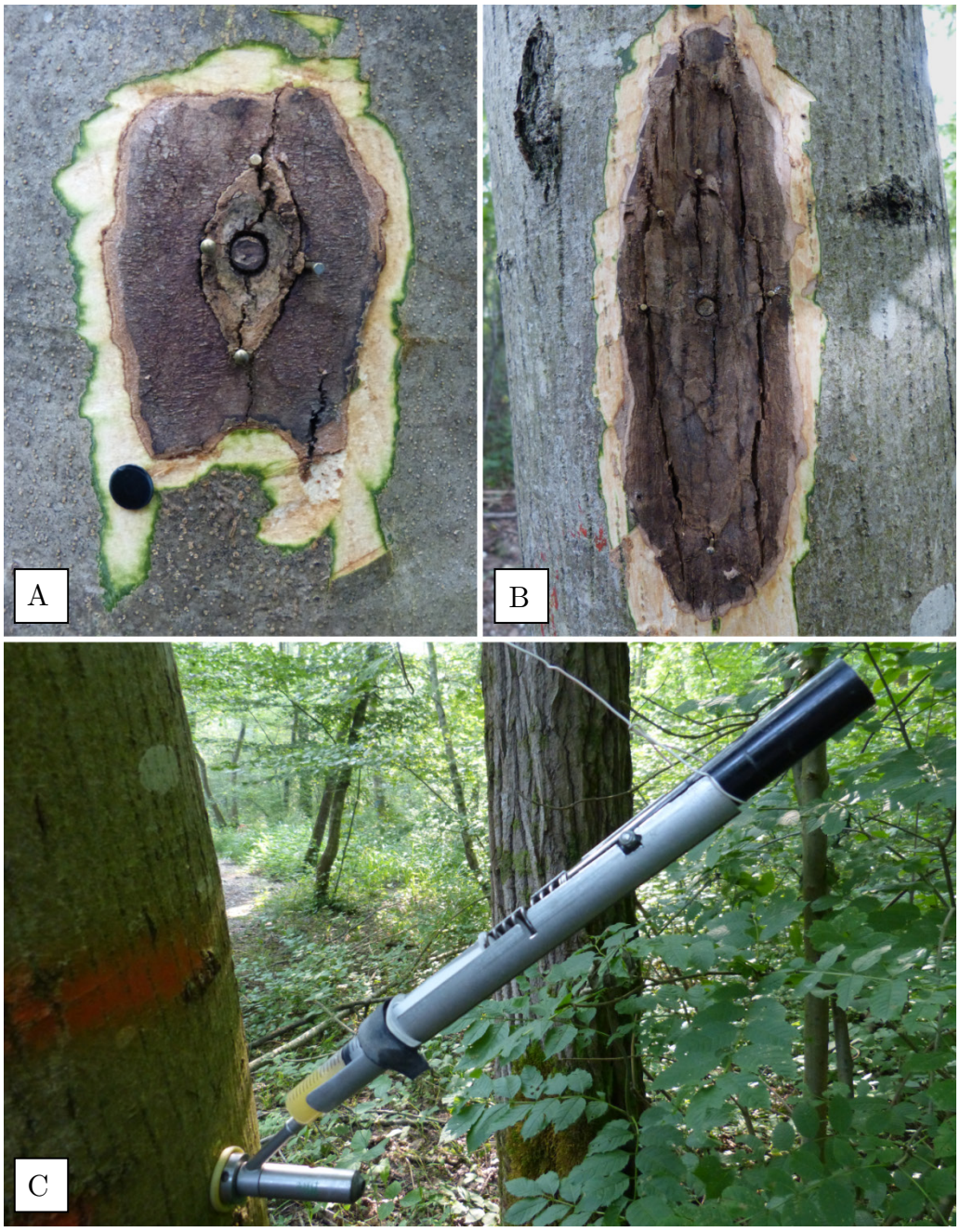
ANNEX 6

R code for bynomial statistic

```
Data <- read.table(file="C:\\Users\\....txt", header=TRUE,
fill=TRUE)
attach(Data)
names(Data)

Y<-cbind(death,total-death)

flm<-glm(Y~dose, family=binomial(link=logit), data= Data)
summary(flm)

pseudoR2<-function(mod) {1-(deviance(mod)/mod$null.deviance)}
pseudoR2(flm)

flp<-glm(Y~dose, family=binomial(link=probit), data= Data)
summary(flp)
pseudoR2(flp)

flc<-glm(Y~dose, family=binomial(link=cloglog), data= Data)
summary(flp)
pseudoR2(flp)

ld50<-function(mod) as.vector(-coef(mod)[1]/coef(mod)[2])
ld50(flm)
ld50(flp)
ld50(flc)
```

ANNEX 7

Endotherapic trial on ashes against ash dieback



In planta evaluation of fungicides. The top two images (A, B) are examples of the necroses developed 482 days after inoculation of *Hymenoscyphus fraxineus*. The bottom image (C) illustrates the tool used for the injection and modifications applied to facilitate long lasting infusion (pictures by Dal Maso E.)

ANNEX 8

Forest Pathology decision on manuscript

Forest Pathology

Preview

From: s.woodward@abdn.ac.uk

To: montecchio@unipd.it

CC:

Subject: Forest Pathology - Decision on Manuscript ID EFP-OA-2014-101.R2

Body: 17-Jan-2015

Dear Prof. Montecchio:

It is a pleasure to accept your manuscript entitled "Large-scale fuzzy rule-based prediction for suitable chestnut ink disease sites: a case study in northeast Italy" in its current form for publication in Forest Pathology. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

Thank you for your fine contribution. On behalf of the Editors of Forest Pathology, we look forward to your continued contributions to the Journal.

Sincerely,
Prof. Stephen Woodward
Editor in Chief, Forest Pathology
s.woodward@abdn.ac.uk

Associate Editor Comments to Author:

Editor
Comments to the Author:
Dera Authorrs, Im pleased to communicate to you that your MS EFP-OA-2014-101.R2 Large-scale fuzzy rule-based prediction for suitable chestnut ink disease sites: a case study in northeast Italy is now ready for pubblication on FP

Reviewer(s)' Comments to Author:

Date Sent: 17-Jan-2015

ANNEX 9

Endotherapic trial on chestnuts against ink disease

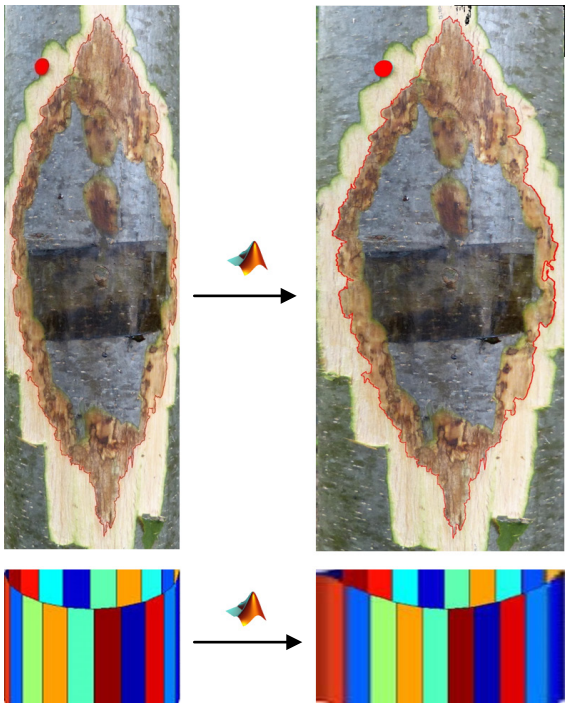
A. The tool (BITE; Montecchio, 2013) used for the injection of potassium phosphite formulations on chestnuts; B, C. The two bottom images are examples of the debarked necroses developed 70 days after inoculation of *Phytophthora cinnamomi* (pictures by Dal Maso E.).

ANNEX 10

Matlab code for cylinder unwrapping

```
I = imread('Image.jpg');
axis on;
box on;
ndims_in = 2;
ndims_out = 2;
f = @(x, unused) sin(x);
g = @(x, unused) asin(x);
inverse_mapping = f;
forward_mapping = g
tdata = [];
tform = maketform('custom', ndims_in, ndims_out, ...
 forward_mapping, inverse_mapping, tdata);
udata = [-0.95 0.95];
vdata = [0 0.00000001];
xdata = [-1.57 1.57];
ydata = [-1 10];
[I2,xdata,ydata]= imtransform(I, tform, 'UData', udata,
'VData', vdata);
imwrite(I2,'ImageTransformed.jpg')
imshow(I2)
```

In the upper part, the script specifically developed for unwrapping of necrotic areas by *Phytophthora cinnamomi* from the chestnut trunk (simplified to cylinder) is reported. On the right, two examples of its application (necroses' pictures by Dal Maso E.).



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