UNIVERSITÀ
DEGLI STUDI DELLA
Tuscia

Department of Plant Protection

PhD thesis in Plant Protection

-CYCLE XXIII-

AGR/12

"Application and validation of an Integrated Control Protocol (ICP) to mitigate ink disease of sweet chestnut (*Castanea sativa* Mill.)."

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Academic period 2007-2011

Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgement has been made in the text.

Selma Franceschini

To my two big loves: family and research…

...hoping to contribute to make this world a little bit better…

INDEX

ACKNOWLEDGMENTS

This work is particularly dedicated to Edoardo who makes my life everyday better and to Alessio who daily collaborates with me to grow up him as he is.

Special thanks are addressed to:

my sisters and my mother who boost me to follow my passions and overcome the difficulties;

Barbara who with me lived this period sharing feelings of satisfactions and dissatisfactions;

the tireless Clara who taught me fundamental lab techniques and helped me every day;

Paola, Sandra e Fulvio for the administrative and sometimes…human…support.

I can't miss to thank also:

Marianna, Giorgio, Natalia, Alessia and Alessandra who helped me in the rare free time which the busy work left to them;

Riccardo for the precious suggestions needed for the elaboration of the ICP;

Donatella, Daniele, Claudio, Andrea, Sig. Felli, Parco Nazionale Monti della Laga which collaborated to the realization of the project through making available their properties for the study.

Special acknowledgements are addressed to:

Professor Andrea Vannini and Anna Maria Vettraino who materially made everything possible and allowed me to have this experience and to understand which are my potentials;

Clive Brasier and Joan Webber who gave me the possibility to experience a wonderful period in their lab and taught me how to carefully assess and process samples and Phytophthora *isolates features and especially to interpret ecological significance of results, lab and field observations.*

This study was undertaken thanks to regional and local projects funded by: the Latium and the Marche Regions, ARSIAL (Azienda Regionale per lo Sviluppo e l'Agricoltura nel Lazio) and Fondazione Varrone.

INTRODUCTION

Castanea spp. is a widely grown plant. Her historical relevance is not only economical but the plant has an environmental, landscaping, cultural and social importance throughout the four continents where the cultivation is practiced.

Italy is the fourth country for the production in the world after Cina, Korea and Turkey. Campania, Calabria and Latium are the main productive Italian regions followed by Liguria and Tuscany. Although a production decline characterized the XX century in the last few decades we assisted to a significant increase of interest for this cultivation (Bounous, 2002; Bounous, 2009). Italy is the second producing country in Europe after Turkey and followed in order by Spain, Portugal, France and Greece (Bonous, 2009).

Since the middle ages nuts of *Castanea sativa* Mill., in Europe and *C. mollissima* and *C. crenata* in Asia provided a dietary staple and, dried, a stored food for the whole year in rural areas. Before the destruction caused by ink disease and chestnut blight, *C. dentata* provided nuts, wood and timber to North-American chestnut growers. Chestnut cultivation has been recently spreading into Southern Hemisphere: Chile Australia and New Zealand has been considered very promising industry (Bounous, 2009).

Castanea sativa Mill. is the species mostly cultivated in Europe, present in 25 countries over a surface of 2 million ha (Conedera *et al*., 2004).

The sustainability of the *Castanea sativa* populations in Europe has been more than once menaced by plant pathogens.

Cryphonectria parasitica (Murr.), the chestnut blight fungus was imported in the 30's from the American continent but before its spread ink disease was considered the primary threat of chestnut survival in Europe.

Ink disease was first recorded in Portugal in 1828 although it is believed that the disease was present in Spain since 1726 (Crandall, 1950).

The disease has been confirmed in France, Spain, Portugal, Great Britain, Slovakia, Hungary, Turkey, Greece and more recently discovered in the Czech Republic (Černy *et al*., 2008; Vannini and Vettraino, 2001; Vettraino *et al*., 2005).

In Italy a disquieting resurgence of the disease was reported at the end of XX century (Vettraino *et al*., 2001).

P. cambivora and *P. cinnamomi* (Rand) are the causal agents of ink disease worldwide. Both species are heterothallic: they demonstrate a well distinct sexual polarity. Although the two species are very similar, there are few morphological and physiological features which enable their identification: the oogonia ornamentation

which is bullate only in *P. cambivora* and the chlamydospores development which is present only in *P. cinnamomi*.

The two pathogens could be also distinct serologically, on the base of proteins and through rDNA RFLP profiling (Erwin and Ribeiro, 1996).

Ecologically the two species display further differences which has to be taken into account in any control measures planning.

Both species are polyphages but *P. cambivora* was reported on a less number of plant species such as *Juglans, Quercus, Malus, Prunus* (Erwin and Ribeiro, 1996).

P. cinnamomi has a temperature range between 12 and 32°C, the mycelia does not tolerate low temperatures, but the pathogen is able to survive at temperature exceeding 32°C through the formation of resistant chlamydopsores. *P. cambivora* is characterized by a wider temperature range (0-32°C and above) but is more easily isolated from soil in autumn and spring with temperatures ranging from 20-27°C and after rainfall precipitations (Vettraino *et al*., 2005).

In the past other *Phytophthora* species, *P. cactorum* (Lebert & Cohn) J. Schröt and *P. plurivora*, as far identified as *P. citricola* (Sawada), were found as soilborne in European chestnut orchards. It has been also demonstrated that these species could be mild pathogenic toward *C. sativa* (Vettraino *et al*., 2001).

Over the years other Phytophthoras were associated to ink disease in Europe. In 2006, *P. pseudosyringae* (T. Jung & Delatour) was found to be the cause of tongue-shaped necrosis of the inner bark and cambium of chestnut in Spain and in 2010 confirmed further in Italy (Pintos Varela *et al*., 2010; Scanu *et al*., 2010). In 2010 *P. cryptogea* (Pethyb. and Laff.) was isolated from chestnut necrotic tissues and soon after reported as further threat of the cultivation in Greece (Perlerou *et al*., 2010).

Phytophthoras propagules spread through active and passive vehicles such as water flow, animals, machinery and humans (Jules *et al*., 2002; Ristaino and Gumpertz, 2000; Vannini *et al*., 2005, Vannini *et al*., 2010).

Root rot incidence caused by *P. cambivora* and *P. cinnamomi* is linked to the waterlogging, soil compaction, organic matter content and fertilization practices (Fonseca *et al*., 2004, Rhoades *et al*., 2004).

It has been demonstrated that landscape features which mainly affect *Phytophthora spp*. inoculum dissemination are creeks and roads densities within affected areas (Jules *et al*., 2002; Jung and Blaschke, 2004). Similarly it has been shown that ink disease severity and plant mortality increase with the increasing proximity to roads and creeks respect

with the disease tends to have a centrifugal diffusion (Natili, 2004; Vannini *et al*., 2005; Vannini *et al*., 2010).

In the light of these epidemiological aspects the management of water flows within ink disease affected orchards is a fundamental point for the control of pathogens spread.

Different methods have been applied over the time to control ink disease. One of them is the "Gandolfo" method which consists in exposing infected tree roots to winter temperature in order to kill the pathogen (Vannini and Vettraino, 2001).

Chemical control of Phytophthoras aims to destroy, inhibit or suppress their growth through the use of fungicides and fungistats either in the field or in the nursery (Erwin and Ribeiro, 1996).

Among protectants the Bordeaux mixture, discovered by Millardet in 1882, was the earliest used for the control of downy mildew caused by *Plasmopara viticola* (Schwinn and Margot, 1991).

The efficacy of copper compounds in the control of *Phytophthora* spp. was also demonstrated for soilborne Phytophthoras and later confirmed for those species associated to ink disease of chestnut (Lucas *et al*., 1990; Coelho *et al*., 2005).

Undoubtedly copper-based fungicides represent the most efficient and economical chemicals to control *Phytophthora* diseases even though they could negatively impact micobiont soil microflora (Graham *et al*., 1986).

Among systemic compounds metalaxyl is the most efficient acylanine active only against *Pythium*, *Phytopthora* and downy mildews (Erwin and Ribeiro, 1996).

Curative effects of acylanine fungicides have been reported for the control of *P. cambivora* in almond and for *P. cinnamomi* on oak (Luque *et al*., 2002; Thomidis and Elena, 2001; Wicks, 1988).

The chemical control of *Phytophthora*-caused diseases could be reached also by the use of phosphonates. Phosphite could be distributed through foliar sprays, soil drenches or plant injections.

Mechanisms of action of phosphonates range from direct fungitoxicity to enhancement of host resistance (Erwin and Ribeiro, 1996; McCarren *et al*., 2009a; McCarren *et al*., 2009b; Walters *et al*., 2005).

It is widely reported that phosphite treatments result in improved canopy health and vigor even though restriction of lesions caused by *Phytophthora* was also demonstrated (Gentile *et al*., 2009; Jung *et al*., 2010; Scott *et al*., 2007; Shearer *et al*., 2006).

Phosphite injections proved to be effective against almond, avocado and grapewine *Phytophthora* diseases (Darrieutort and Lecomte, 2007; Fernàndez-Escobar *et al*., 1999; Wicks and Hall, 1990; Whiley *et al*., 1992).

Potassium phosphite trunk injections revealed to have a persistent effect over the time on *Eucalyptus* species based on what reported by Shearer *et al*., (2006).

Recently K-phosphite foliar sprays and trunk injections were efficiently applied to control ink disease of chestnut caused both by *P. cinnamomi* and *P. cambivora* in pots and field trials (Gentile *et al*., 2009).

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AIMS

Since the second half of 2004 the Department of Plant Protection (DiProP-University of Tuscia-Viterbo) has been elaborating an Integrated Control Protocol (ICP) to mitigate the effects of the ink disease of sweet chestnut.

In 2005 the application of an ICP including management of water flow, fungicides and copper sulphate applications and endotherapic treatments led to a reduction of plant mortality in a small chestnut orchard located in Caprarola (Viterbo-Italy) (Vannini *et al*., 2009).

Thanks to these positive preliminary results the DiProP developed a series of regional research pilot projects addressed to set up the best ICP protocol to control ink disease of chestnut in Central Italy (Vettraino *et al*., 2010).

Such protocols has also to take in account *Phytophthora* population monitoring within the time and the space through a preliminary assessment in terms of number of species and pathogen inoculum pressure and a final assessment of the control protocol efficacy.

This PhD thesis inserts in these projects and therefore their aims coincide with those of the projects. The thesis was divided in four chapters which represent the objectives of the research and a final fifth chapter which summarizes the conclusions to which these three years of activities led to.

Aims of the present PhD thesis are:

1. to individuate a reliable assay to monitor living *Phytophthora* spp. soilborne in ink diseased chestnut orchards, developing a standard baiting technique allowing multiple sample analyses to facilitate extensive surveys;
2. to characterize resident soilborne *Phytophthora* spp. population in the infected chestnut sites and interpret their epidemiological and ecological significance;
3. to determine the insurgence of metalaxyl-resistant isolates of *P. cambivora* after fungicide field treatments;
4. to exploit the knowledge on the control of *Phytophthora* spp. available from the literature and collected during these years of experience to enhance, apply and validate the ICP for the control of ink disease as far elaborated from the DiProP in view of small and large scale applications.

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CHAPTER I

An improved baiting technique to recover and quantify *Phytophthora* spp. from chestnut soil samples

Key word*s*: *Castanea sativa*, *Phytophthora* detection and isolation

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Abstract

Baiting is a simple and specific method to detect Phytopthoras from environmental samples. It represents an important stage of diagnostic process to be completed before the definition of any disease control strategies.

This study was undertaken to assess the efficacy of different biological baits to isolate and quantify soilborne Phytophthoras in ink diseased chestnut areas. Azalea, *Castanea* and *Rhododendron* leaves, and carnation petals were tested to bait Phytophthoras from soil samples incubated at 10 and 20°C. Isolation percentage differed significantly between incubation temperature with highest value for 20°C.

Baits showed to be differently selective toward *Phytophthora* species. Carnation petals and *Rhododendron* leaves were most effective for the isolation of rare Phytophthoras while the use of chestnut leaves and/or carnation petals received the highest *Phytophthora* infection rate. An improved baiting method for the isolation of *Phytophthora* species in chestnut soil samples was therefore elaborated and adopted to monitor Phytophthoras population within chestnut experimental areas as described in Chapter II.

Introduction

The genus *Phytophthora* is known to be potentially harmful to woody plants in natural forests and hardwood plantations. Some species are host specific such as *Phytophthora quercina* on oaks (Jung *et al*., 2000*)*. Other Phytophthoras are associated with decline of a broad range of hosts in forest stands, wildlands and plantations. Several Phytophthoras have been associated to ink disease one of the main threats of sweet chestnut in Italy and Europe (Vettraino *et al*., 2001) even though the ecological significance of their finding has still to be clarified. *P. cambivora* and *P. cinnamomi* were the only isolated species from chestnut necrotic tissues since 2006 when also *P. pseudosyringae* was found to be the cause of chestnut stem necrosis in Spain (Pintos Varela *et al*., 2010) the pathogenicity of this species has been recently confirmed in Italy by Scanu *et al*. (2010). Recently also *P. cryptogea* has been isolated from chestnut tissues in Greece and proved to be aggressive against *Castanea sativa* seedlings (Perlerou *et al*., 2010).

Further *Phytophthora* species were found to inhabit chestnut orchard soils in Central Italy such as *P. nicotianae, P. megasperma* and *P. sansomeana* (see Chapter II of this thesis). Among them *P. megasperma* were able to cause significant root rot on one year chestnut seedlings.

The growing scientific interest in Phytophthoras disease, phylogeny and ecology resulted in the last decade in a sensible intensification of surveys activities aiming to study population structure, local biodiversity and to describe new invasive or native species (Hansen *et al*, 2009; Jung and Burgess, 2009; Jung *et al*., 2011). Highly reliable detection methods represent key tools to achieve these objectives.

Even though the rise of molecular techniques have been widespreading and fully exploited to readily diagnosis *Phytophthora* species in different kind of environmental samples such as water, soil and plant tissues (Hayden *et al*., 2006; Ippolito *et al*., 2004; Schubert *et al*., 1999), baiting is still a simple and specific method for the detection of these pathogens and even used to confirm results from other detection methods and last but not the least, to prove the inoculum vitality (Cooke *et al*., 2007; Pettitt *et al*., 2000). Indeed the most part of the studies on *Phytophthora* populations still rely partially or totally on baiting as technique to detect and isolate these pathogens. Therefore peculiar baitings, often combined with PCR techniques, has been developed to isolate and/or quantify different species of *Phytophthora* among which *P. alni* and *P. cinnamomi* and *P plurivora* (ex *P. citricola*) , *P. quercina* and *P. erythroseptica* (Eden *et al*., 2000; Nawayakkara *et al*., 2010; Nechwatal *et al*., 2001; Streito *et al*., 2002).

The advantages of using baiting for Phytophthoras detection is that it is simple and cost effective allowing to recover isolates for further phenotyping and genotyping studies required to discover possible hybridization events between species and even the rising of new species.

The disadvantages of the technique are represented by the low sensitivity comparing to other molecular-based detection methods such as DAS-ELISA, nested end point PCR and TaqMan real time PCR (Vettraino *et al*., 2010) which on the other hand may be subject to false positives.

Indeed a combination of either culturing and molecular methods was suggested to overcome peculiar techniques limits to diagnose *Phytophthora ramorum* presence on multiple natural hosts (Vettraino *et al*., 2010).

This study aims to increase the sensitivity of baiting to isolate and quantify the presence of Phytophthoras in chestnut soil samples through the utilization of different baits. The influence of the incubation temperature on *Phytophthora* spp. isolation was also evaluated in order to individuate the most favorable for the recovery of sweet the chestnut pathogen *P. cambivora* as well as the highest number of other Phytophthoras.

Materials and methods

Baits efficacy

Soil samples were collected from the rizosphere of chestnut plants as well as from forest roads and natural drainages and creeks bordering or crossing the orchards. Samples were brought to the laboratory and processed within 24 hrs. Soils were gently mixed and a 200ml aliquot of each sample was flooded with approximately 450ml of tap water. Debris were carefully removed from the water surface. Azalea and *Castanea* leaves, *Rhododendron* disks and carnation petals were utilized as baits. Three baits each species were used (figure 1).

Baits were removed when necrotic on more than 30% of the surface, usually after 48-72hrs of incubation.

Each single bait was washed twice in sterile distilled water to remove soil particles and dried on paper towel. Baits were sectioned into eight pieces and plated on Selective Malt Agar (Malt-Extract 15g/l, Agar 20g/l, Pimaricin 0.01g/l, Sodium-Ampicillin 0.25g/l, Hymexazol 0.05g/l; Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate 95% 15mg/l).

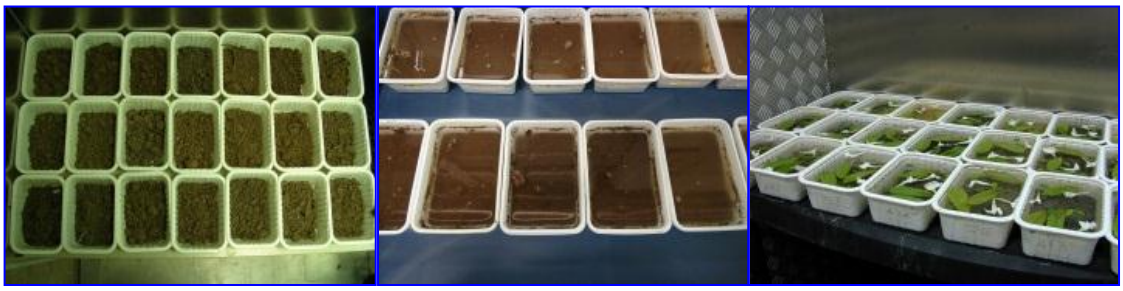

Figure 1. Images of soil baiting set up of a set of chestnut soil samples conducted in the climatic chamber.

After one week all baits were removed and plated as stated before. *Phytophthora* colonies emerging from baits pieces were subcultured on Carrot-Agar (200g/L sliced carrots, 20g/l Agar). *Phytophthora* isolates were identified on the basis of their morphological and molecular traits (Erwin and Ribeiro; 1996, Martin and Tooley, 2003; see also Chapter II of this thesis).

Incubation temperature and timing

To determine the influence of the temperature and the day of baits withdrawal on *Phytophthora* spp. and especially *P. cambivora* isolation a further experiment was

carried out incubating five soil samples at 10 and 20°C and taking all the baits off after three and five days from the beginning of the experiment. Five azalea, *Rhododendron* and *Castanea* leaves disks of 10 mm of diameter and 5 carnation petals were used and sectioned in four parts for plating. Only carnation petals were sectioned in eight pieces (figure 2). Each sample were incubated in two replicates and processed at the same time. The experiment was repeated after air drying of soil samples for two weeks.

Figure 2. Single box prepared for the baiting assay used to determine the influence of the temperature and the length of the incubation period on *Phytophthora* spp. and *P. cambivora* isolation.

Statistical analysis

Data were elaborated with GraphPad Prism 4.0 (GraphPad Software, San Diego, USA). When Fisher's exact test was performed the percentage of isolation was reported in the graph to make results easier to read.

Results

Baits efficacy

The use of different baits allowed to highlight significant differences ($P>0.05$) in their efficacy to capture Phytophthoras inoculum: *Castanea* leaves and carnation petals detected the highest number of isolates with an average percentage of 24 and 22% respectively (figure. 3).

Results of a number of baiting assays carried out over a two years period allowed to individuate carnation petals and *Rhododendron* disks as the most efficient baits to describe Phytophthoras biodiversity detecting even rare species. In fact up to nine and eight different species were isolated with carnation petals and *Rhododendron* disks

respectively. The only exception was *P. cryptogea* which was isolated with azalea but *Rhododendron* (table I).

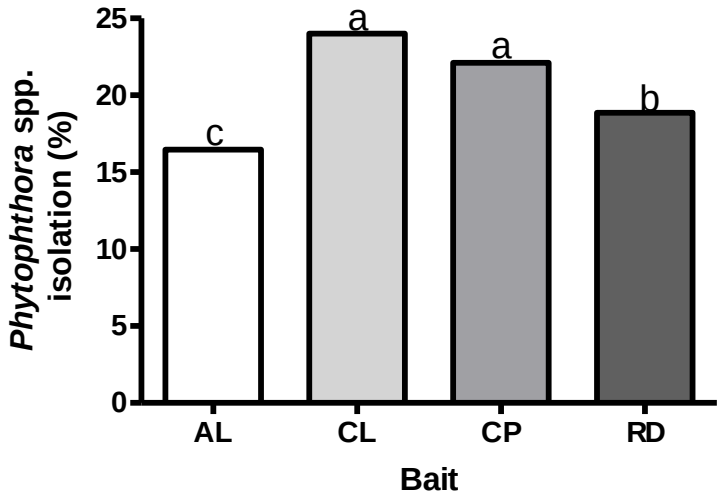

Figure 3. Percentage of isolation of *Phytophthora* spp. for each bait: AL= azalea leaves; CL= chestnut young leaves; CP= carnation petals; RD=*Rhododendron* disks. Different letters above bers indicate significant differences (P<0.05) between groups (Fisher's exact test).

Table I. *Phytophthora* species isolated for each bait type. Data shown by this table are a review of a set of baiting experiments conducted on chestnut soil samples over a period of two years from 2007.

***Phytophthora* sp.**	**AL**	**CL**	**CP**	**RD**
P. cactorum	+	+	+	+
P. cambivora	+	+	+	+
P. cinnamomi	+	+	+	+
P. cryptogea	+	+	+	-
P. gonapodyides	+	+	+	+
P. megasperma	-	-	+	-
P. nicotianae	-	-	-	+
P. plurivora	+	+	+	+
P. pseudosyringae	-	-	+	+
P. sansomeana	-	-	+	-
P. syringae	-	-	+	+

Incubation temperature and timing

Phytophthora spp. isolation didn't differ significantly (P>0.05) between replicates and no differences were found between days of baits withdrawal within each incubation temperature and bait type. Significant differences (P<0.001) between the incubation

temperature were found. Twenty degrees centigrade incubation resulted in 42.1% of *Phytophthora* spp. isolation compared to 7% obtained at 10°C (figure 4).

Statistical analysis also revealed that the 10°C incubation temperature resulted in isolation datasets not normally distributed probably due to the high frequency of putative false negatives.

An extremely low level of *Phytophthora* spp. isolation (12 isolates on a total of 800 baits pieces plated) was obtained after air drying soil samples. The only species recovered at that time was *P. plurivora*.

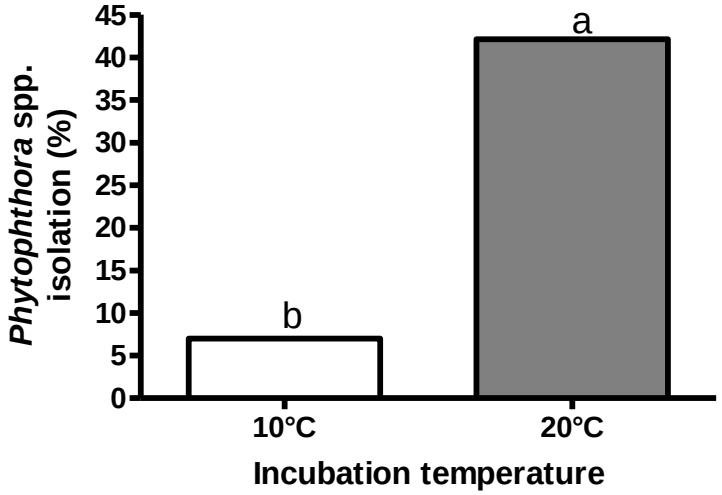

Figure 4. *Phytophthora* spp. recovery at 10°C and 20°C temperature of sample incubation. Different letters above columns indicate significant differences P<0.0001 between mean of values (Mann Whitney test).

The temperature of 20°C and an incubation period of five days allowed to recover three species of *Phytophthora* (*P. cambivora*, *P. plurivora* and *P. pseudosyringae*), while two species (*P. cambivora* and *P. plurivora*) were obtained after three days incubation. Twenty degrees and five days of incubation were the experimental conditions which enable to isolate a significantly (P<0.0001 Fisher's exact test) higher number of *P. cambivora* isolates (table II).

Table II. Number of positive isolation of *Phytophthora cambivora* for each day of withdrawal and each incubation temperature.

	Third day		Fifth day	
Replicate	T10	T20	T10	T20
A	27 (25)	69 (39)	21 (19)	51 (23)
B	34 (34)	42 (28)	38 (36)	76 (40)
Tot	61	111	59	127
Replicate mean	30,5	55,5	29,5	63,5

Numbers in brackets refer to isolates obtained from carnation petals plating.

Discussion and conclusions

Many plant diseases have been discovered to be caused by a number of previously undescribed *Phytophthora* species which are currently threatening natural as well as forestry ecosystems. The monitoring of Phytophthoras is hence a crucial point to elaborate control strategies against such type of plant pathogens. With the increasing plant movement which followed the globalization process another fundamental goal of the pathogen monitoring is to early detect new pathogens in semi natural and natural environment to reduce the risk of epidemics (Brasier, 2008).

Among conventional methods for the detection of Phytophthoras from water, soil and tissue samples serial dilution technique and baiting are still widely used (Cooke *et al*., 2007). The first one is not effective when the inoculum density is relatively low (<10 propagules per gram of dry soil) which is likely to often occur in naturally infested soils (Tsao, 1983). A variety of seedlings, plant parts or fruit are currently used as baits; in general specific baits are used to detect and/or quantify specific Phytophthoras; two well-known examples are represented by lupin radicles for *P. cinnamomi*, oak leaflets for *P. quercina* (Eden *et al*., 2000; Nechwatal *et al*., 2001). It is likely that the selectivity of baits could constitute a limit of the technique in the detection of multiple *Phytophthora* species present in the same sample (Erwin and Ribeiro, 1996). Furthermore it is evident as European forest soils are inhabited by a large number of invasive *Phytophthora* species (Brasier, 2000; Jung *et al*., 2000; Vettraino *et al*., 2005) whose ecology, epidemiology and reciprocal interactions have still to be determined. Thus, efficient baiting protocols for multiple *Phytophthora* detection is a paramount need in such investigations.

However this study was undertaken also to maximize the sensitivity for *P. cambivora* detection, that is considered in Italy the main causal agent of ink disease.

Similar was the reason which leaded in 2000 Eden *et al*., to carry on a very accurate work to determine an efficient method for the quantification of *Phytophthora cinnamomi* in soil.

Results obtained from the present study demonstrate that the use of multiple bait types achieves both the objectives that are, the study of soilborne Phytophthora biodiversity, and *Phytophthora cambivora* quantification.

Azalea young leaves, *Rhododendron* disks and carnation petals were found to be the best combination of baits to estimate *Phytophthora* diversity. Even though chestnut leaves were also effective in the recovery of a high number of *Phytophthora* isolates we suggest to use carnation petals because they are available for the most part of the year rather than chestnut leaves which are obtainable from the field during spring and summer only. If the intention is to use chestnut leaves a nursery of *Castanea sativa* seedlings has to be maintained to guarantee the availability of those baits all over the year.

The incubation temperature of 20°C was found to enhance isolation quality and quantity and it was also the temperature that enable to obtain normal distributed data for the statistical analyses. It is not surprising that this temperature, close to *P. cambivora* optimum, led to the recovery of a highest number of pathogen isolates.

In accordance to Eden *et al*., (2000) air-drying soil samples led to a sensible decrease of the *Phytophthora* spp. isolation probably due to propagules death.

After air drying soil samples no *P: cambivora* and no *P. pseudosyringae* isolates were recovered.

The recovery of *P. plurivora* but not *P. pseudosyringae* from dried samples was probably due to the fact that *P. plurivora* was the prevalent species as determined with baiting of fresh soil samples.

The lower abundance of *P. cambivora* respect to *P. plurivora* and the homothallism could be the reasons why after air drying soil samples *P. plurivora* but not *P. cambivora* was recovered. Oospores are indeed known to be more resistant to desiccation respect the other *Phytophthora* structures (e.g. mycelia, sporangia and chlamydospores,) which could also be present in infested soils.

The work carried out over these year of experimentation allowed us to give the following recommendations for isolating and quantifying *Phytophthora* spp. from a large number of chestnut soil samples (≤100).

To maximize the sensitivity of baiting collect samples soil (at least 1l volume) from around the trees after removing the litter at a depth of 10-20cm. Process soil samples as soon as possible after the collection.

Before aliquot samples in plastic boxes mix them gently without sieving.

Flood the samples with tap water to reach the top edge of the boxes. A good volume will be 200ml of sample in a 500ml plastic boxes flooded with approximately 450ml of tap water.

Remove raw particles carefully from the water surface and leave the samples overnight to allow soil decantation. Put 3 carnation petals, 3 *Rhododendron* disks and 3 azalea young leaves on the water surface.

Incubate chestnut soil samples at a temperature of 20°C with a photoperiod of 12hours, possibly in a climatic chamber.

The soil:water ratio and the incubation conditions are of particular importance in the case of inoculum quantification.

Remove baits when the necrosis is approximately over the 30% of bait surface which normally is reached at the third day of baiting. Check sample daily for further bait necrosis and remove them if needed until the seventh day. Leave baits for additional 2 days and at the tenth day plate symptomatic and asymptomatic baits only if quantification is the aim.

Surface disinfection of baits before plating which was so far reported to reduce isolation success (Montgomerie and Kennedy, 1983) was omitted in the present study. The non-significant bacterial contaminations observed on the selective medium was attributed to the careful drying of baits after the two washing steps.

After plating, checking baits daily for a period of one week allow to isolate slower growing species.

If the objective of the experimentation is limited to quantify *P. cambivora* inoculum it is suggestible to use only five carnation petals as baits, 20°C as incubation temperature remove all baits after 5 days of incubation.

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CHAPTER II

Phytophthora species inhabiting ink diseased chestnut stands in Central Italy

Key words: *Castanea sativa*, Phytophthoras , soilborne pathogens

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Abstract

As a consequence of global climatic change and global trade the soilborne *Phytophthora* complex in ink diseased chestnut stands could underlie some changes in biodiversity and behavior to chestnut trees increasing the risk of invasiveness. Understanding the ecology of the complex of resident Phytophthoras in different chestnut stands and the patterns of their dispersal is of particular importance in order to produce effective control protocols to reduce the impact of ink disease and to detect further *Phytophthora* pathogens which could constitute a threat toward natural and semi-natural ecosystems.

Surveys of ink diseased chestnut stands were conducted to obtain an overview of the *Phytophthora* spp. soil inhabiting population. Plant rhizosphere, rural roads and creeks were sampled to study species distribution within the areas. Species were identified on the basis of their morphological features and confirmed by sequencing ITS rDNA. Eleven *Phytophthora* species were recovered. The presence of at least one of the two casual agents of the ink disease (*P. cambivora* (Petri) Buism or *P. cinnamomi* Rand) was ascertained in all sites surveyed. *P. nicotianae, P. megasperma* and *P. sansomeana* were recovered along with previously detected Phytophthoras: *P. cactorum*, *P. gonapodyides* and *P. plurivora*, *P. pseudosyringae* and *P. syringae*. Most of the species were isolated from rural roads and plant rhizosphere but also from creeks samples. The ecological significance of *Phytophthora* spp. spatial distribution within ink diseased chestnut stands is discussed.

Introduction

During the past decades many information accumulated regarding the detection and the spread of soil- and air-borne-*Phytophthora* spp. which are currently threatening a number of woody species in natural and semi-natural ecosystems. Alder, beech, *Eucaliptus*, larch, and oak are just few examples of the ability of *Phytophthora* to cause devastating diseases toward forest trees species worldwide (Brasier, 2000; Brasier *et al*., 2004; Jung *et al*., 2003; Jung *et al*., 2004; Webber *et al*., 2010; Hansen, 2000; Hardy, 2009).

In Europe a number of invasive *Phytophthora* species have been wide spreading through forests and semi-natural plant communities being *P. alni*, *P. cinnamomi* and, more recently, *P. lateralis* and *P. ramorum*, the four most worrying pathogens threatening native and introduced hosts (Brasier, 2000; Robin *et al*., 2010; Webber *et al*., 2010).

From southern UK to Greece *P. cambivora* and *P. cinnamomi* are responsible for the so-called ink disease of sweet chestnut (*Castanea sativa* Mill.) (Brasier, 2000; Černy *et al*., 2008; Vannini and Vettraino, 2001). Both pathogens cause chlorosis, microphylly, root and collar rot and plant death. It was recently demonstrated that disease severity of ink disease as well as plant mortality increase as a consequence of the proximity of plants to creeks and roads (Vannini *et al*., 2005; Vannini *et al*., 2010) which must therefore be seriously taken into account during the elaboration and application of any disease control strategies (Vannini *et al*. 2009; Vettraino *et al*., 2010, see also Chapter III and IV of this thesis).

The *Phytophthora*-complex inhabiting Italian and European chestnut stands were investigated in the last decade (Vettraino *et al*., 2001 and 2005) leading to the recovery of eight species among which one was not identified. To date in Italy *P. cactorum*, *P. cambivora*, *P. cinnamomi*, *P. gonapodyides*; *P. plurivora* (ex *P. citricola*), *P. pseudosyringae* and *P. syringae* are the species detected on sweet chestnut stands (Scanu *et al*., 2010; Vettraino *et al*., 2001; Vettraino *et al*., 2005).

This study was undertaken to describe the current composition of *Phytophthora* spp. population inhabiting soils of three ink diseased chestnut stands in Central Italy using baiting procedure developed in Chapter I, and to map their spatial distribution in a heterogeneous landscape typical of chestnut areas in Italy (Vannini *et al*., 2010). Results of this study were taken in account to plan management strategies for the disease control described in Chapter IV.

Materials and methods

Sites and soil characteristics and sampling

To investigate *Phytophthora* spp. composition and distribution in chestnut groves, soil surveys were conducted in three experimental areas located in Central Italy chosen for the application of the Integrated Control Protocol.

Two sites (A and B) are located in the Latium region in the Viterbo and the Rieti Province respectively while site C is found in Ascoli Piceno (Marche region) (see also Chapter IV of the thesis).

The three sites differ in extension, elevation, geomorphology, management practices and soil type (for more information regarding the three sites see also Chapter IV of the thesis).

Soil characteristics including also pH, texture and percentage of organic matter were analyzed and listed in table I.

To study the pattern of their distribution within the time and the space soil samples were collected from plant rhizosphere, rural roads and creeks (table II) from 2007 through 2010. About a hundred and fifty samples were collected in the three areas investigated at each sampling time.

Table I. Chemical properties of soils per each experimental area where Phytophthoras monitoring was conducted.

	Site A Viterbo	**Site B Rieti**	**Site C Ascoli Piceno**
pH in water	6,88	5,5	6,44
clay (%)	30,8	16,2	12,5
sand (%)	47,5	65,4	70,9
loam (%)	21,7	18,4	16,6
conductivity (microSiem/cm)	260	390	330
organic matter (%	2,51	1,11	3,68
N tot (%)	0,12	0,07	0,23
ppm P	69,7	79,5	14,6
ppm K	587	154	140
ppm Ca	1771	582	1397
ppm Mg	277	155	255
ppm Na	52	37	40
Mg/K	1,51	3,22	5,83
Cations exchange capacity (%)			
K (%)	11,7	8,3	3,7
Mg (%)	17,7	26,8	21,8
Ca (%)	68,9	61,5	72,7
Na (%)	1,8	3,4	1,8

Table II. Type and number of samples for each of the four chestnut sites investigated.

Chetsnut grove location	**Plant rhizosphere**	**Creeks**	**Rural roads**
Arola (Marche Region)	39	6	10
Rieti (Latium Region)	55	1	2
Carbognano (Latium Region)	34	0	1
Total	**128**	**7**	**13**

Soil baiting and isolation

Soil samples were processed by baiting (Jung *et al*., 1996) using carnation, chestnut, azalea and rhododendron as baits (see also Chapter I of this thesis).

Baiting assays were carried out for ten days in a climatic chamber (20°C; 12h photoperiod). Baits were removed at the development of necrosis and plated on Selective Malt Agar (Malt-Extract 15g/l, Agar 20g/l, Pimaricin 0.01g/l, Sodium-Ampicillin 0.25g/l, Hymexazol 0.05g/l; Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate 95% 15mg/l).

Plates were daily checked for *Phytophthora* growth. *Phytophthora*-like hyphae were sub-cultured on Carrot-Agar (CA) prepared at 20% of concentration for the identification.

Phytophthora identification

Phytophthora colonies were assessed for their main morphological features: colony patterns on CA and PDA (Potato Dextrose Agar), sporangia type, hetero- or homo-thallism (Erwin and Ribeiro, 1996). DNA of representative isolates of each morphotype was obtained from liquid culture grown on Carrot juice (20% concentration) (Brasier *et al*., 2010).

Molecular identification was carried out by sequencing ITS1, 5.8S and ITS2 rDNA repeat using primers ITS6 and ITS4 (Cooke *et al*., 2000; Martin and Tooley, 2003). Sequences were edited with BioEdit Software (BioEdit, version 7.0) (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html) (Hall, 1999) and compared with those existent on NCBI database.

In order to further confirm the identification of *Phytophthora* species recovered in chestnut areas in Central Italy ITS sequences of one representative isolate of each species (table III) were aligned with other ITS sequences (five each species) downloaded from the NCBI database (table VIII). Additional eight *Phytophthora* species (*P. alni, P. asparagi, P. hedraiandra, P. cinnamomi* var. *parvispora, P. citricola* authentic type, *P. drechsleri, P. foliorum* and *P. palmivora*,) were added to highlight species differences. ITS sequence of *Phythium ultimum* was used as outgroup. Clustalw2 alignment was analyzed with PHYLYP software (Felsenstein, 1993). The robustness of DNADIST Tree was tested through 1000 BS trial. TREEVIEW was used to drawn the phylogenetic tree (Page, 1996).

Pathogenicity assays

Pathogenicity tests were conducted for those *Phytophthora* species for which was not published the aggressiveness to *C. sativa*.

Colonies were maintained on CA at 18°C and subcultured monthly. Plugs taken from the margin of actively growing colonies of each *Phytophthora* species (i.e. *P. megasperma*, *P. nicotianae, P. pseudosyringae,* and *P. syringae*) were used to inoculate sterilized millet grains moistened with carrot-juice (1.5 w:v). Moistened millet grain inoculated with CA represented the control. Inoculum was grown at 18°C for 3 weeks in the dark. Thirty ml of infested grains were used to inoculate potting soil (0.06 v:v) of one years old seedlings of *C. sativa.*

Plants were watered as needed and flooded every two weeks. After 3 months plants were removed from potting soil, roots washed with tap water. Small segments of fine roots were plated on SMA to isolate *Phytophthora* spp. inoculated. The root apparatus was divided in diseased and healthy and oven-dried at 100°C.

Data analysis and statistic

Phytophthora recovery percentages were expressed as total of pieces which were positive to the isolation on the total of pieces plated.

The significance ($P<0.05$) of linear regressions between soil features, as reported in table I, and *Phytophthora* spp. isolation percentages were analyzed through GraphPad Prism 4.0 (GraphPad Software, San Diego, USA)

Data of pathogenicity assays were elaborated with GraphPad Prism 4.0 (GraphPad Software, San Diego, USA). After the verification that data were normally distributed ANOVA analysis was carried out. $P<0.01$ was used as level of difference significance.

Images elaboration

To map the distribution of sweet chestnut pathogens within areas positional data of plants, road and creeks were acquired through GPS (Leica Geosystems®). Thematic map shown was constructed using Arc-View 3.1 (Environmental Systems Research Institute, ESRI, Inc., Redlands, CA, USA) software.

Results

Phytophthora *spp. recovery and pathogenicity*

A total of 11 *Phytophthora* species has been recovered from soil samples from the three sites. The code of representative isolates are listed in table II, their alignment with ITS sequences downloaded from the NCBI database confirmed the correct identification the species (figure 2 and table VIII).

Four *Phytophthora* species (*P. cactorum*, *P. cambivora*, *P. gonapodyides*, *P. plurivora*) were common to all three sites (table III). *P. crytpogea* was common to site A and B, *P. pseudosyringae* to site A and C, *P. megasperma* and *P. syringae* were detected in site B and C. *P. nicotianae* and *P. sansomeana* were recovered only once in site A and C respectively. *P. cinnamomi* was isolated in a chestnut site close (less than 1 Km apart) to Site A in an extra survey in 2009 before his confirmation in the coastal area of the Latium Region soon after.

P. cactorum, *P. cambivora*, *P. gonapodyides* and *P. plurivora* were the species most frequently recovered along the year in site B and C (table IV to VI) while *P. pseudosyringae* was isolated more frequently in site A and only once in site C.

P. cambivora, *P. cactorum*, *P. gonapodyides*, *P. plurivora* and *P. syringae* were isolated from all type of soil samples (table VII). These species, with the exception of *P. syringae*, were also the most frequently recovered during the year (table IV to VI). The same pattern of recovery was confirmed also for *P. cinnamomi* in a coastal area close to Rome (Vettraino personal communication).

P. pseudosyringae was isolated from rhizosphere soil and from rural road but not from creeks samples. *P. megasperma* and *P. sansomeana* were found only in roads.

P. nicotianae was the only species isolated exclusively from rhizophere but not the other types of soil samples (table VII).

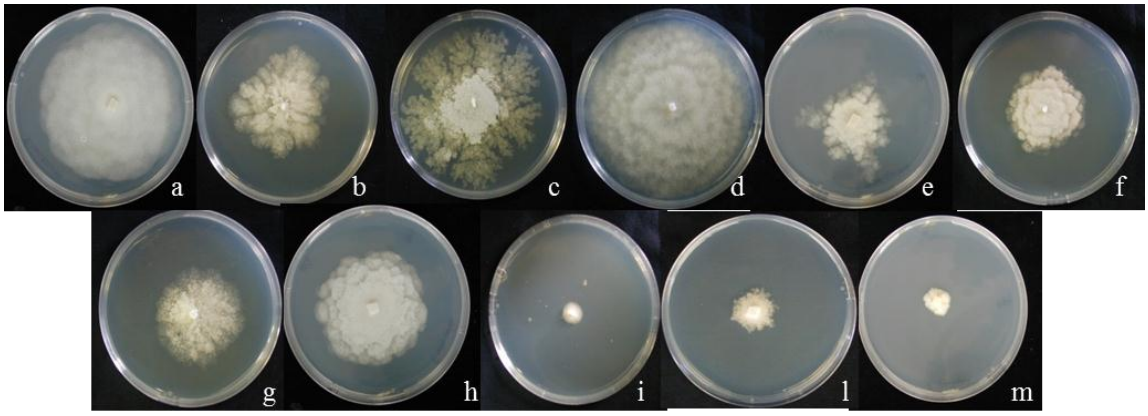

Figure 1. PDA morphology of *Phytophthora* species isolated: a) *P. cactorum*; b) *P. cambivora*; c) *P. cinnamomi*; d) *P. cryptogea*; e) *P. gonapodyides*, f) *P. megasperma*; g) *P. nicotianae*; h) *P. plurivora*; i) *P. pseudosyringae*; l) *P. sansomeana*; m) *P. syrinage.*

Table III. Isolate codes of *Phytophthora* species identified per each site assessed. Isolates were those used for ITS sequencing.

N°	***Phytophthora* species**	**Isolate name**	**Site**
1	*P. cactorum*	M3AM	A,B,C
2	*P. cambivora*	7T6	A,B,C
3	*P. cinnamomi*	79AG5	A
4	*P. cryptogea*	P8T1	A,B
5	*P. gonapodyides*	M5AM	A,B,C
6	*P. megasperma*	3T0	B,C
7	*P. nicotianae*	M1T16C	A
8	*P. plurivora*	M3T9	A,B,C
9	*P. pseudosyringae*	BR1	A,C
10	*P. sansomeana*	M7AM	C
11	*P. syringae*	M1BS	B,C

Table IV. Temporal distribution of *Phytophthora* species for site A in the Viterbo Province. Grey-colored cells indicate the positive recovery of the *Phytophthora* species at each sampling time.

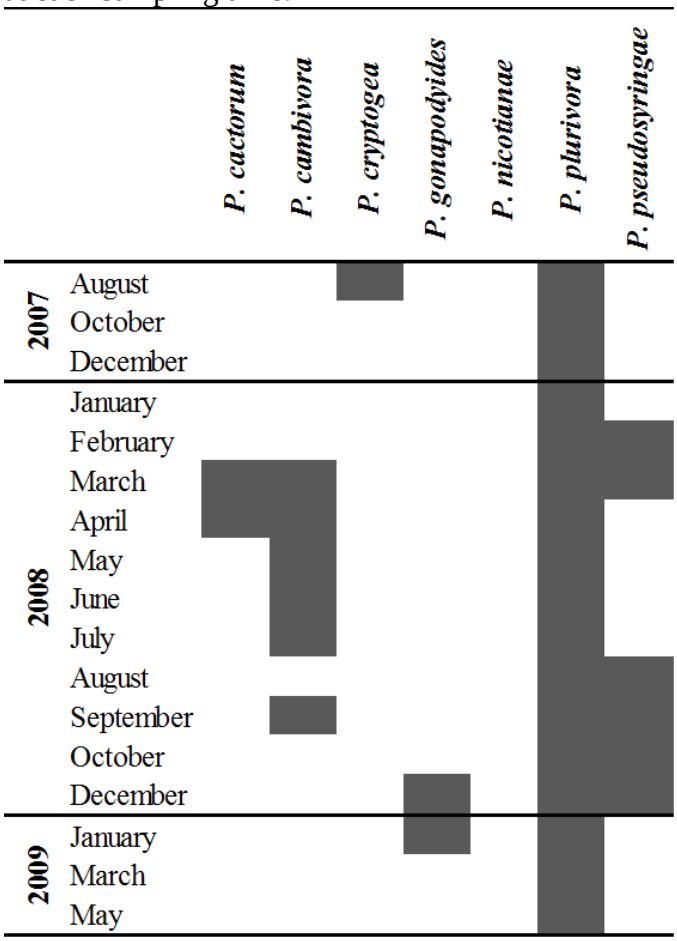

Table V. Temporal distribution of *Phytophthora* species for site B in the Rieti province. Grey-colored cells indicate the positive recovery of the *Phytophthora* species at each sampling time.

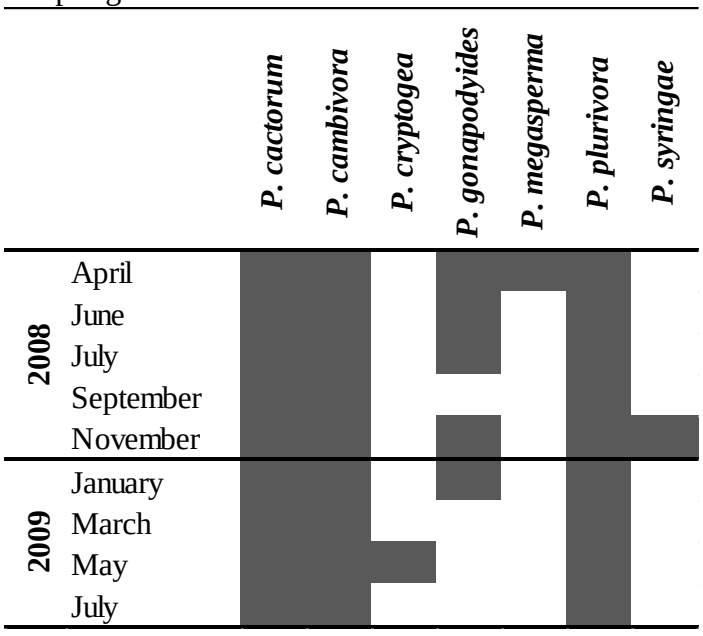

Table VI. Temporal distribution of *Phytophthora* species for site C in the Ascoli Piceno province. Grey-colored cells indicate the positive recovery of the *Phytophthora* species at each sampling time.

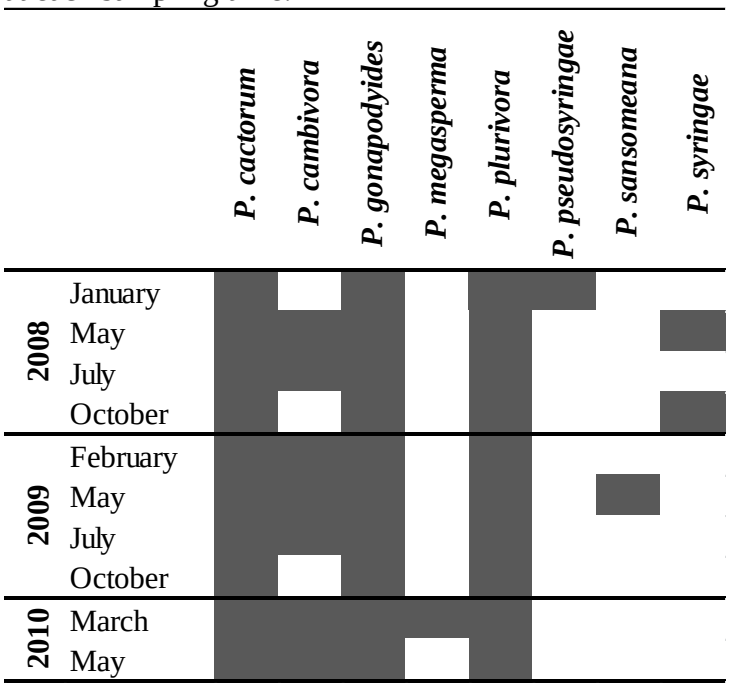

Table VII. Kind of sample from which each *Phytophthora* species were recovered.

n°	***Phytophthora species***	***Plant rhizosphere***	***Creeks***	**Rural roads**
1	*P. cactorum*	+	+	+
2	*P. cambivora*	+	+	+
3	*P. cinnamomi*	+	*	*
4	*P. cryptogea*	-	-	+
5	*P. gonapodyides*	+	+	+
6	*P. megasperma*	-	-	+
7	*P. nicotianae*	+	-	-
8	*P. plurivora*	+	+	+
9	*P. pseudosyringae*	+	-	+
10	*P. sansomeana*	-	-	+
11	*P. syringae*	+	+	+

* roads were not present in the area surveyed.

Table VIII. ITS sequence accession numbers from GenBank of *Phytophthora* isolates used for the phylogenetic analyses.

n°	***Phytophthora* species**	**Isolate**	**Origin**	**GenBank accession number**
1	*P. cactorum*	PFE3	Italy	FJ183724
2		753	Poland	EU240182
3		K423-05	USA	EF052680
4		94f	Hungary	EU594603.1
5		89-375 18S	Switzerland	EU106586.1
6	*P. cambivora*	I-111003	USA	HM004220
7		301.07	Poland	FM199985.1
8		M305918	Japan	AB367381.1
9		ISPaVe1950	Italy	AM269752.1
10		96-201	Switzerland	EU000156
11	*P. cinnamomi*	CMW19404	South Africa	DQ988173
12		P3232	USA	GU594781
13		96-601	Switzerland	EU000160
14		94006	Taiwan	AY251660
15		PC17	Spain	EF150876
16	*P. cryptogea*	1072	Colombia	EU200283.1
17		CIPH	Italy	HM627524.1
18		STE-U6261	South Africa	GU191214
19		TSOK 1	Greece	GQ428329.1
20		VHS14620	Australia	EU301142.2
21	*P. gonapodyides*	P1047	Germany	AF541889
22		P897	Tasmania	AF541888.1
23		P1057	France	AF541887
24		P149	UK	AF541892
25		I_2B4L	USA	HM00423
26	*P. megasperma*	VHS17183	Australia	EU301166.2
27		P1277	Scotland	AF541898.1
28		85	Switzerland	EU194390.1
29		P1279	France	AF541896
30		P476	USA	AF541897.1
31	*P. nicotianae*	DB 11032009	Italy	FJ843100.1
32		LP1	Cina	GU299778.1
33		VHS16099	Australia	EU301135.2
34		WPC:1153C903	USA	GU259148.1
35		PS-21	Spain	EU244849.1
36	*P. plurivora*	IMI342898	UK	AF266789
37		PLU92	Turkey	FJ665231
38		CBS124093	Germany	FJ665225
39		39A8	USA	GQ303153
40		TARI24355	Taiwan	GU111599
41	*P. pseudosyringae*	CST2A	Italy	GU460375.1
42		III_2-1P	USA	HM004228.1
43		ATCC MYA-4222	Germany	FJ196755.1
44		Fera 20907761 cc2655	UK	FJ971898.1
45		473	USA(CA)	AY369374.1
46	*P. syringae*	RHS 10715/01	UK	AY611644
47		II_2-4P	USA	HM004229.1
48		PS-66	Spain	EU244851.1
49		89-342	Switzerland	EU000117.1
50		AG5 clone 1	Argentina	AY787034.1
51	*P. alni*	P772	UK	AY689133
52	*P. asparagi*	VHS17644	Australia	EU301168.2
53	*P. citricola* (authentic type)	CBS295.29	Japan	FJ560913.1
54	*P. cinnamomi* var. *parvispora*	COR1	Italy	GU460376
55	*P. drechsleri*	PS-43	Spain	EU194428
56	*P. foliorum*	-	USA	EF120469.1
57	*P. hedraiandra*	P12445	Spain	EF174430
58	*P. palmivora*	K8r	Iran	HM582095.1
59	*Phythium ultimum*	PID 190	USA	EU147492.1

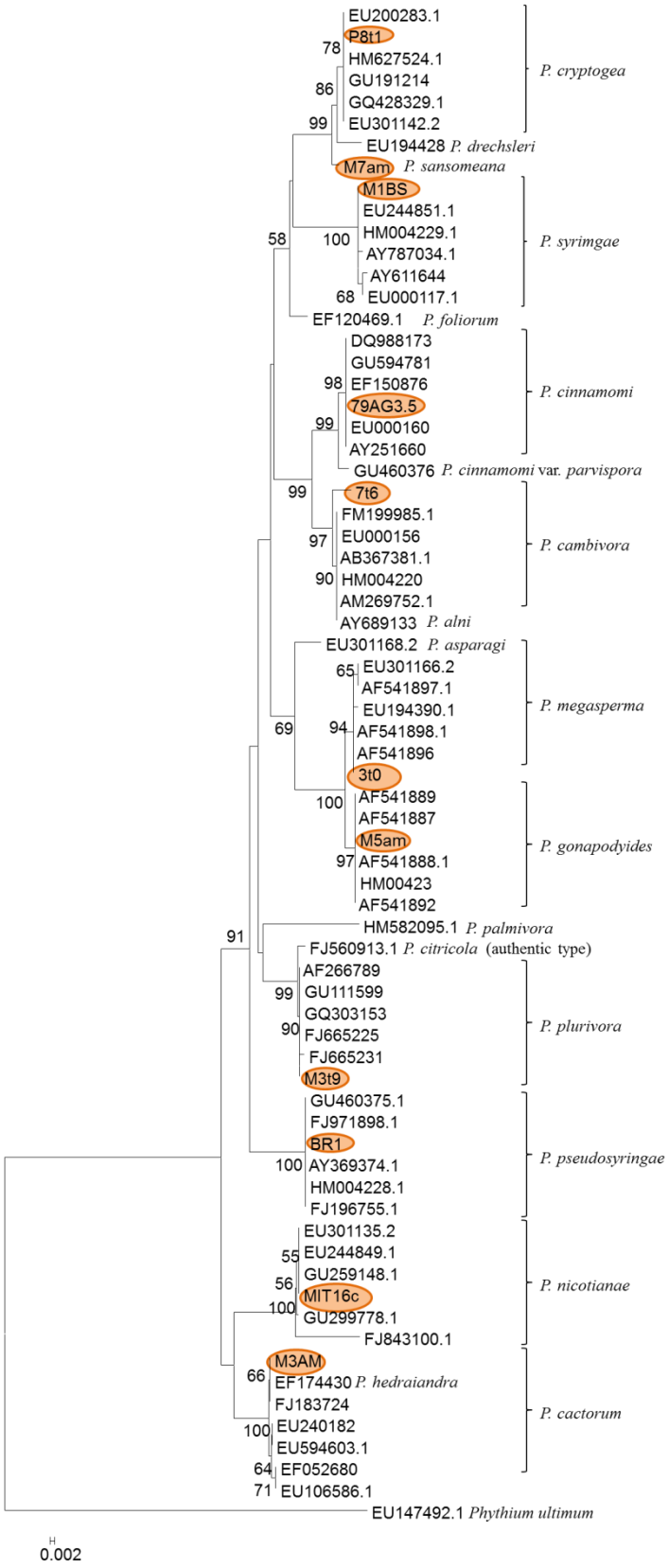

Figure 2. Phylogram of ITS sequences alignment of *Phytophthora* representative isolates with ITS of overseas Phytophthoras. Numbers at node indicate bootstrap values, only bootstrap values above 50 were shown. Sequences highlighted are isolates of table II. Number at the bottom of the phylogram indicate substitution sites scale.

Phytophthora spp. inoculum in plant rhizosphere increases during the late spring in site B and C with the highest peaks often occurring in May (data not shown).

The general average of *Phytophthora* spp. isolation percentage in plant rhizophere ranged from 6% to 41%. It was highest (20%) in site A respect to site B and C were the same rate was respectively 17% and 16.5%.

Site C was characterized by the highest recovery of *P. plurivora* which reached the maximum in June 2008 with a percentage of 48%.

P. cactorum was isolated more frequently in site C but with a major abundance (33.5 %) in site B.

P. cambivora was isolated infrequently in site A and with a highest rate in site B.

Concerning the analyses of the relationships between isolation percentages and soil properties significant positive linear regressions were found between *P. cactorum* isolation and exchangeable Mg (P=0.02) and *P. plurivora* isolation and loam content (P=0.01).

After two weeks from inoculation with *Phytophthora* spp. chestnut seedlings showed similar symptoms to what developed in the field (chlorosis and apical bud collapse) (figure 3b). No symptoms were observed for the plants inoculated with un-infested millet grains (figure 3a). *P. megasperma* was the only species among Phytophthoras tested leading to the development of significant root rot (P<0.01) respect to the control (figure 3c and 4). It was also the only species re-isolated from diseased root.

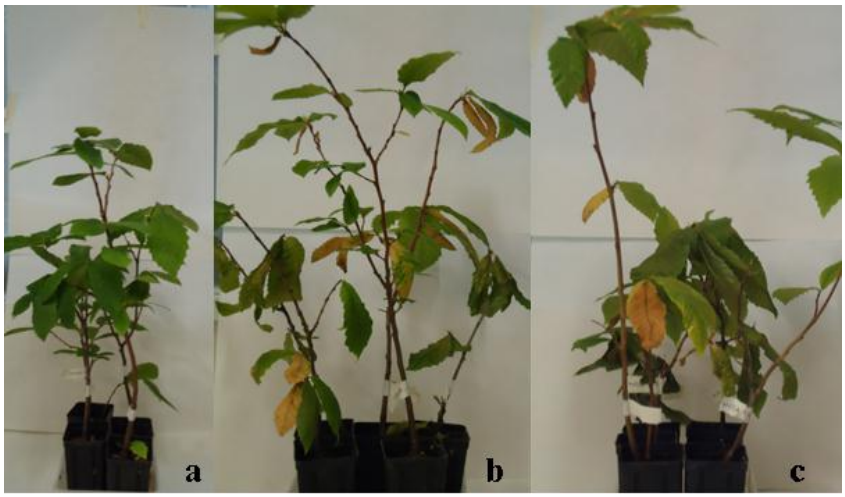

Figure 3. Symptoms on chestnut seedlings inoculated with *Phytophthora* species. a) control; b) plants inoculated with *P. cambivora*; c) plants inoculated with *P. megasperma*.

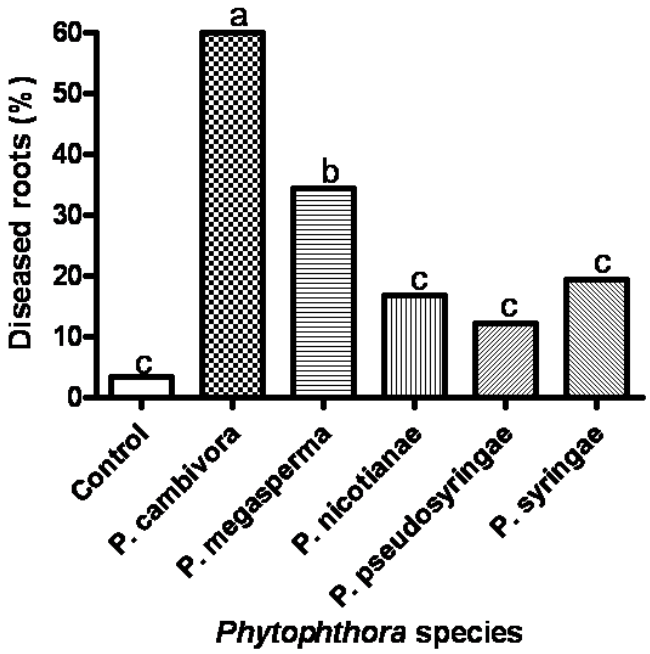

Figure 4. Percentage of diseased root (dry weight) caused by each *Phytophthora* species on one years old *C. sativa* seedlings. Different letters at the top of each column indicate significant differences P<0.01 between mean of values (ANOVA).

Phytophthora *spp. distribution maps*

Maps generated in SIT environment show the spatial distribution of *Phytophthora* species within the areas.

An example of the elaboration for *P. cambivora* within ink diseased stands is reported in figure 5.

Similar maps could be generated for any of the *Phytophthora* species detected and indeed could be used to visualize their distribution and when updated in real time they could be useful to follow the pattern of their spread within studied areas.

Figure 5 was chosen in particular to underline the pattern of distribution of *Phytophthora cambivora* which is evidently diffuse on the whole creek and road nets within site C. This site was also chosen to study *P. cambivora* inoculum along paths and natural channels over the years because of the highest abundance of roads and creeks.

It was found that the chestnut pathogen inoculum was highest in the late spring in both natural drainages and rural pathways (figure 6).

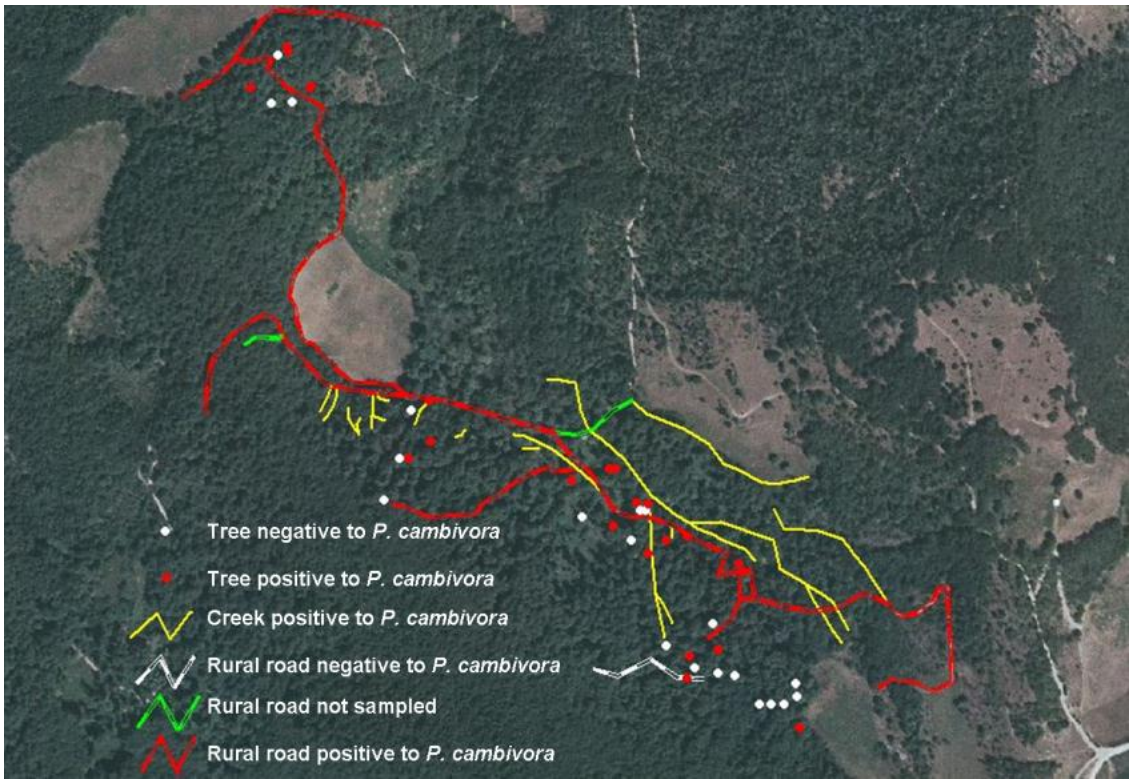

Figure 5. Thematic map of site C (Marche Region) showing the spatial distribution of *Phytophthora cambivora* within the area. Results refer to the whole sampling period from January 2008 to May 2010.

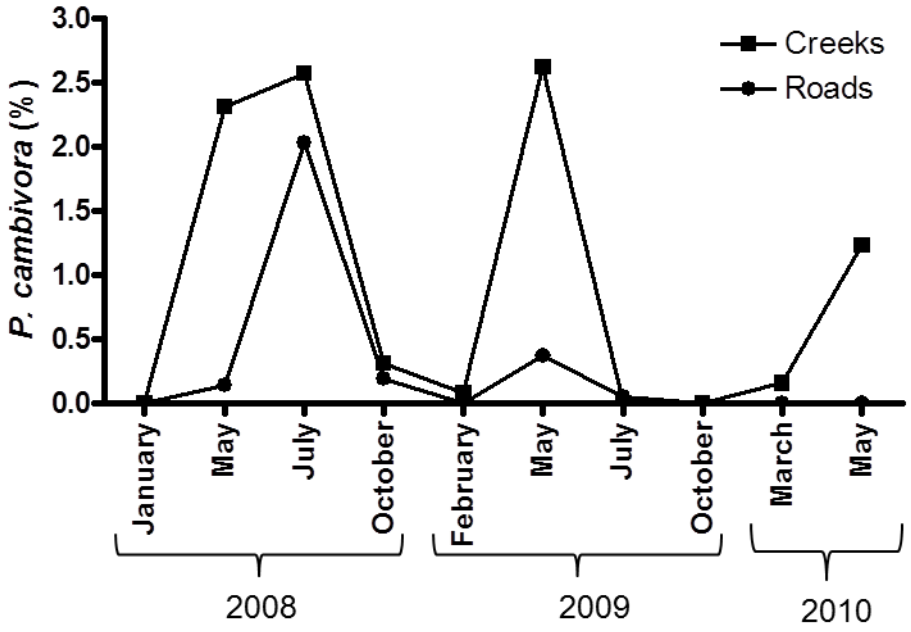

Figure 6. *P. cambivora* inoculum variation along rural roads and creeks in Site C (Ascoli Piceno province) during the period 2008-2010.

Discussion and conclusions

The population of resident Phytophthoras in chestnut stands in Central Italy is abundant and variable. Neighbour-joining tree supported the correct identification of the species reported in this study.

P. cambivora, *P. plurivora*, *P. cactorum* and *P. gonapodyides* were found to be the most common as already reported by Vettraino *et al*., (2005) in Europe. *P. plurivora* demonstrated to be the most abundant and persistent species in chestnut soils along the year confirming its association to woodlands in Europe (Jung and Burgess, 2009). However the European origin of this species is still controversial since its recent record from native forests in remote Himalayan areas (Vannini unpublished).

Differently to what reported by Vettraino *et al*., (2001), *P. gonapodyides* was here reported to be present also in the soil collected around trees. Probably this species reached the rhizophere of plants through soil particles carriage by water flows within the area (site C).

Among the other species *P. nicotianae* is a new record in chestnut areas. The species is indeed commonly associated to agricultural systems rather than to forest typologies. This is also the first report of *P. cryptogea* in Italian chestnut groves. The presence of this last species in chestnut soils is important as it has been recently demonstrated its pathogenicity against *C. sativa* in Greece (Perlerou *et al*., 2010). We also reported for the first time the presence of *P. sansomeana* in Europe. Before our detection this species has been isolated from Douglas fir, soybean and corn (Tang *et al*., 2010; Zelaya-Molina *et al*., 2010; Hansen *et al*., 2009) in America and Asia. Its pathogenicity toward chestnut has still to be evaluated.

Particularly alarming is the finding of *P. cinnamomi* in chestnut stands in Central Italy. The pathogen was isolated from a site in Carbognano (Viterbo province) in 2009 but more recently *P. cinnamomi* was found to be the cause of ink disease foci in a further region in Central Italy close to the seaside in the Rome province (Vettraino personal communication). *P. cinnamomi* inoculum possibly entered chestnut stands from plantations (e.g. walnut) and other forest typologies (e.g. oak) (Vettraino *et al*., 2002) which found at lower altitude. It is likely that in Italy the pathogen is moving to higher altitudes possibly in association to the increase of winter temperatures (Brasier, 2000; Vettraino *et al*., 2005) that may facilitated inoculum overwintering in soil.

Moreover the detection of several *Phytophthora* species and especially *P. cambivora* on rural roads and creeks deserves particular consideration especially during control

protocols elaboration (Ristaino and Gumpertz, 2000; Jung *et al*., 2004; Vannini *et al.*, 2010). Indeed this finding strengthen the importance of limiting the soil particle movements through any kind human activity and especially the excessive machinery transit within the field.

P. cambivora inoculum along rural roads and natural creeks was found to be particularly abundant during the spring-summer period.

Most of *Phytophthora* spp. reported in the present study were isolated from samples collected in roads and creeks which therefore are confirmed as important corridors for Phytophthoras inoculum representing a menace for surrounding plantations, not just toward chestnut (Jules *et al*., 2002; Vannini *et al*., 2005; Vannini *et al*., 2009; Vannini et al., 2010; Weste, 1975).

Indeed pathogenicity tests allowed to individuate *P. megasperma* as a possible new threat for chestnut as it led to a significant root rot on incolulated chesnut seedlings.

To our experience *P. pseudosyringae* was unable to cause significant root damage to *C. sativa* seedlings even though this pathogen was reported to be aggressive to chestnut if stem inoculated (Pintos Varela *et al*., 2007; Scanu *et al*., 2010). It is therefore important to strengthen the need to uniform techniques to screen pathogenicity ability of this type of plant pathogen (Vannini personal communication).

Along with the recent report of *P. cryptogea* in Greece (Perlerou *et al*., 2010) and the demonstrated ability of *P. pseudosyringae* of causing stem necrosis on sweet chestnut (Pintos Varela *et al*., 2007; Scanu *et al*., 2010) *P. megasperma* increases the number of Phytophthoras potentially harmful toward *C. sativa* and highlight once again the need of further studies to clarify the role of those *Phytophthora* species in the disease development.

The detection of other *Phytophthora* species such as *P. nicotianae* represents a strong evidence of the anthropic disturbance of and poses disquieting thoughts on the "free" movement of invasive Phytophthoras among croplands, woodland and nurseries typical in Italy as well as in most of the European countries (Brasier and Jung, 2006; Brasier, 2008; Rizzo *et al*., 2005).

Under an ecological point of view several main questions come to the mind after overviewing the results of this study: why so many *Phytophthora* species on chestnut orchards? How do they behave in the rhizosphere? Are they competing or acting together? Why so many *Phytophthora* species detected in rural roads and creeks?

Together with changing environmental factors one possible ecological explanation of the detection of so many *Phytophthora* species on Italian chestnut orchards is linked to the fact that they are "human-disturbed" environment. In undisturbed environment it is likely to recover one or a few *Phytophthora* species from the same soil sample (Brasier personal communication). On the contrary in disturbed environment anthropic influence allows the introduction of multiple species through the nursery supply and this is probably the reason why the same species are found in different European country. Once introduced Phytophthoras proliferate and one species can out-perform, making its detection easier, depending on different site factors alike the geological substrate, soil texture and pH (Jung *et al*., 2000). Indeed, to our experience, in chestnut soils *P. plurivora* prevails in the clay and compacted soil with pH 6.9 (site A), *P*: *cambivora* was isolated more frequently abundantly from the sandy-clay soil in site B (pH 5.5); *P. cactorum* w in site B but was more frequently detected in C where soil pH is 6.4 pH and the texture is sandy.

How do these organisms behave in the rhizosphere? If we assume that *Phytophthora* zoospores do not compete at root level but, on the contrary, interspecific signals promote plant infection, (Kong *et al*., 2010) the probable ecological explanation is that when multiple species found in the rhizosphere their zoospores cooperate to suppress plant defenses but once in the tissue the well adapted species to the host tissue environment, prevails.

To answer to the last main question that this study pose (Why so many *Phytophthora* on rural roads?) a possible explanation would be because *Phytophthora* zoospores accumulate where the human disturbance is higher alike paths and roads and subsequently they move into creeks through natural water flows. It is likely that the same species found in streams and/or roads also found in the rhizosphere but at lower concentration which could make pathogens individually undetectable through the use of conventional techniques (see also Chapter I of this thesis). Indeed an highest level of *Phytophthora* diversity in stream could be reached by the adoption of the filtration techniques rather than baiting (Smith *et al*., 2007).

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CHAPTER III

Metalaxyl-resistant isolates of *Phytophthora cambivora* recovered from treated chestnut orchards

Key words: fungicide resistance, ink disease, EC_{50}

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Abstract

Phytophthora cambivora were recovered from the rhizosphere soil of chestnut plants in an experimental area located in the Rieti province in Central Italy. Isolates were collected before and after metalaxyl field treatments. Sensitivity to the fungicide was tested through measuring isolates growth rate performance of on V8-juice Agar amended with increasing concentration of metalaxyl. It was found that after two series of field treatments about a half of isolates recovered from the treated area displayed fungicide resistance. The results of the present work must be taken into account by who is interested in *P. cambivora* control especially ink disease control.

Introduction

The use of phenylamides against *Phytophthora* pathogens is a widely adopted control measure (Erwin and Ribeiro, 1996).

Metalaxyl treatments were early studied for the control of *Phytophthora infestans* (Bruck *et al*., 1980). The authors successfully demonstrated that the fungicide was effective in suppressing mycelial development, sporangia sporulation and disease symptoms (lesions leaves).

Efficacy of metalaxyl treatments, both as soil drenches and stem paints, were than demonstrated for *P. cinnamomi* on avocado seedlings in glasshouse (Snyman and Kotzé, 1984). Later the fungicide effectiveness was confirmed for *P. cactorum* and *P. citrophthora* on peach trees and for *P. cactorum* on apple trees both through laboratory and field experiments (Boughalleb *et al*., 2006; Thomidis and Elena; 2001; Thomidis and Tsipouridis, 2001; Thomidis, 2002).

Shortly after that metalaxyl was introduced to control *Phytophthora* diseases, fungicide resistance appeared on a widespread scale. The phenomena, firstly reported for *P. infestans* (Davidse *et al*., 1981; Dowley and O'Sullivan; 1981) has been reported, among others, for field isolates of *P. capsici* and *P. nicotianae* (Parra and Ristaino, 1998; Timmer and Graham, 1998).

It has been demonstrated that the resistance to phenylamides in *P. infestans* became established in A1 populations before the appearance of A2 type and that resistant isolates of the pathogen express equal or greater fitness than sensitive isolates, but no correlation was detected between resistance and race structure (Gisi and Cohen, 1996). Soon after studies on the genetics of *P. infestans* metalaxyl resistance found a dominant

pattern of the phenomena guided by a single locus with a major effect and the alleles being dominant or recessive (Lee *et al*., 1999).

The fungicide metalaxyl applied as drench had a good efficacy in the control *P. cambivora* that infect almond (Wicks, 1988). Similar results was soon after reported for the control of ink disease of chestnut in Greece (Skoudridakis and Bourbos, 1990), but to date no works have been done to determine the possible rising of fungicide resistance on the sweet chestnut pathogen *P. cambivora*.

Recently field treatments with metalaxyl have been adopted within the application of an Integrated Control Protocol to manage ink disease of *Castanea sativa* in Central Italy (Vannini *et al*., 2009; Vettraino *et al*., 2010).

The aim of the present work was evaluate the effect of treatments effectuated on 2008 and 2009 on the pathogen. The poisoned agar technique was chosen to determine the insurgence of resistant strains among a set of field isolates collected after one and after two cycles of the acylanine applications.

Materials and Methods

Twenty *P. cambivora* A2 isolates were tested to determine their sensitivity to different concentrations of metalaxyl. Isolates were collected from a local population in an experimental area in the Rieti Province (see Chapter IV for more information) where metalaxyl treatments were applied in two consecutive years in August 2008 and May 2009. *P. cambivora* isolates were selected among those recovered from rhizophere soil samples of chestnut plants before the treatments (5 isolates) after the first treatment (3 isolates) and after the second treatment (12 isolates). The sensitivity of isolates was assessed on V8 Juice Agar (V8A: 100 ml/l V8 juice; 16 g/l Agar; 3 g/l $CaCO_3$) amended with increasing metalaxyl concentration: 0.1, 1, 10, 100 µg/ml). Metalaxyl-amended V8A was prepared and 14 ml of media was poured for each plate and stored at 4°C until needed. Colonies of the twenty *P. cambivora* isolates were grown on non-amended V8A for six days at 23°C in the dark. Six mm plugs from the edge of each colony were transferred on the metalaxyl-amended V8A plate for each concentration. Two replicates were prepared for each isolate and for each concentration. Two plates of non-amended V8A were also inoculated with each isolate as control. Amended and non-amended dishes were incubated at 23°C in the dark.

Colony diameter was measured in two directions for each individual dish, averaged and compared to average colony diameter from non-amended media. The percentage growth

of each isolate relative to the non-amended control was plotted against the $\log_{10}$ of metalaxyl concentration. Colony diameter increment values expressed as a percentage of the non-amended controls were plotted against $\log_{10}$ of metalaxyl concentration to give a regression line for each isolate (Parra and Ristaino, 2001). The effective concentration (EC_{50}: the point on the regression line at which 50% of isolate growth is inhibited) for each isolate was calculated. Isolates were classified in three classes on the base of the EC_{50} values: $EC_{50} \leq 5$µg/ml sensitive; 5 µg/ml $< EC_{50} \leq 10$µg/ml tolerant and $EC_{50} > 10$ µg/ml resistant. Graphs were drawn with GraphPad Prism 4.0 (GraphPad Software, San Diego, USA).

Results

Growth inhibition at increasing metalaxyl concentrations for each of the three group isolates assessed are displayed in figure 1.

Linear regression between the colony increment and the metalaxyl concentration were calculated for each isolate. R^2 values varied from 0.57 of isolate 20 to 0.98 for isolate 6. EC_{50} values varied from 0.00057 µg/ml of isolate 20 to 441 µg/ml of isolate 8. Only for isolate 6 EC_{50} was very high: 7943 µg/ml which also correspond to the highest value of R^2. Isolate 5 had an intermediate EC_{50} of 7.13µg/ml and was hence classified as tolerant (table I).

None of the five *P. cambivora* isolates recovered from chestnut rhizophere before and after the first field treatment resulted resistant or tolerant to metalaxyl.

Five metalaxyl-resistant and one metalaxyl-tolerant isolates were found among the ones recovered after the second field treatment made on 2009.

Figure 2 shows plates of the *P. cambivora* sensitive (isolate15), tolerant (isolate5) and resistant (isolate 6) to metalaxyl at the end of the growth rate experiment.

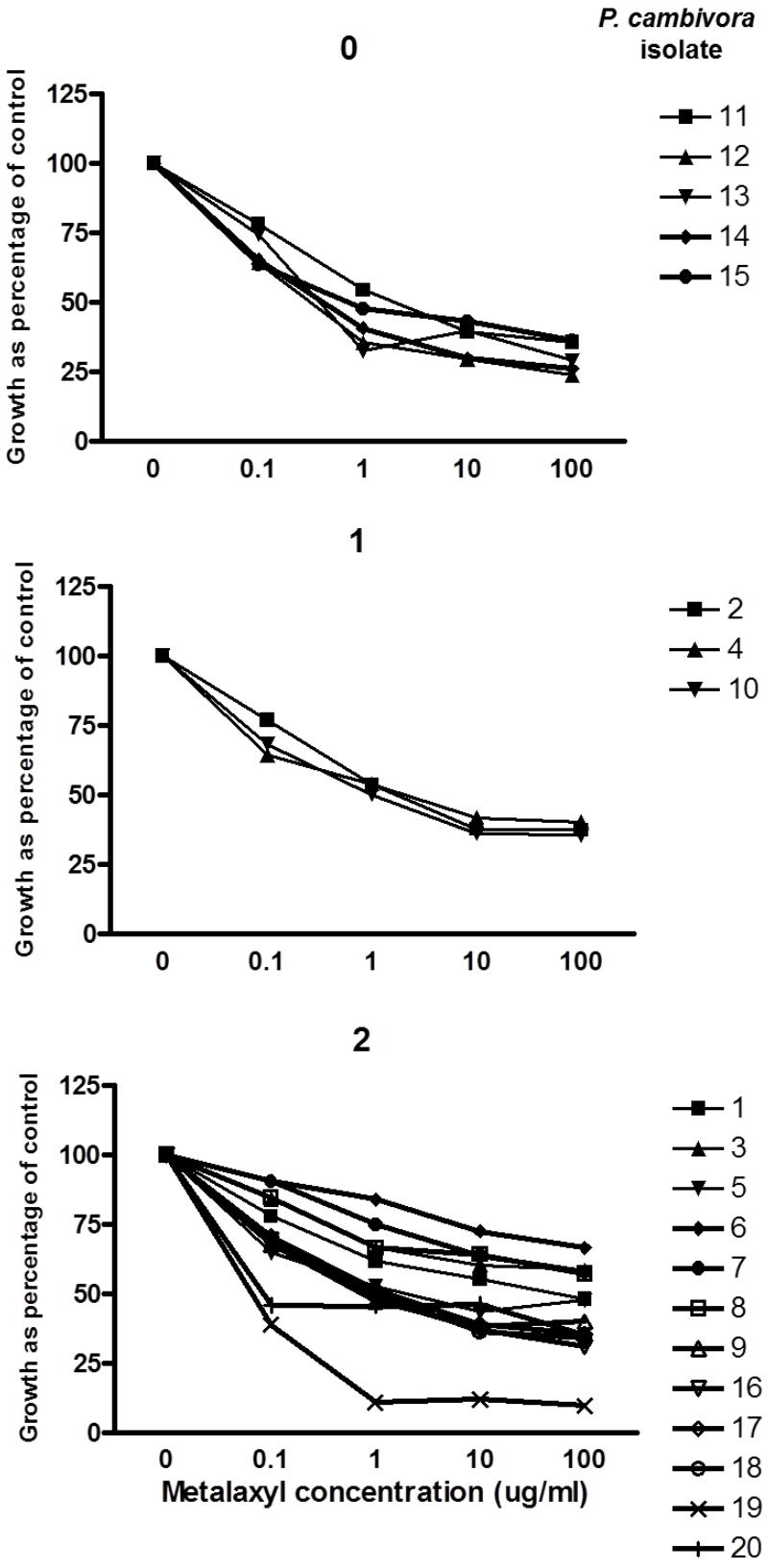

Figure 1. Growth as percentage of the control of *P. cambivora* isolates at increasing concentration of metalaxyl. Isolates were grouped based on the number of metalaxyl field treatments they received: from the top to the bottom: 0,1 and 2.

Table I. Degree of sensitivity to metalaxyl of *P. cambivora* strains isolated from chestnut field before and after metalaxyl treatments.

***P. cambivora* isolate**	**Number of metalaxyl field treatments**	**EC_{50} (µg/ml)**	**Classification**
1	2	43,26867	resistant
2	1	4,09394	sensitive
3	2	412,41839	resistant
4	1	3,24698	sensitive
5	2	7,13016	tolerant
6	2	7.943,98238	resistant
7	2	317,26805	resistant
8	2	440,88995	resistant
9	2	2,10231	sensitive
10	1	1,93251	sensitive
11	0	4,35385	sensitive
12	0	0,37723	sensitive
13	0	1,06350	sensitive
14	0	0,58169	sensitive
15	0	1,73632	sensitive
17	2	1,65386	sensitive
18	2	2,72385	sensitive
19	2	1,97186	sensitive
20	2	0,00057	sensitive
21	2	0,02384	sensitive

Figure 3. *P. cambivora* isolates after the trial on metalaxyl-amended media. Number on the top of each photo are isolate numbers. Media concentration (µg/ml) is indicated on the corner of each plate for each isolate.

Discussion and Conclusions

Among phenylamide fungicides metalaxyl is one of the most efficient compound to control *Phytophthora* pathogens. The application methods include spray, drench, dip, granules and seed dressing. The fungicide application may lead to the emergence of resistant strains and this was widely demonstrated for *Phytophthora infestans* but also for other species as well (Erwin and Ribeiro, 1996).

The genetic of metalaxyl resistance is still not fully understood even though in *P. infestans* is apparently guided by a major gene (Lee *et al*., 1999).

Metalaxyl was successfully applied to control *P. cambivora* on almond (Wicks, 1988) and there is a report on their efficacy on the control of ink disease of sweet chestnut (Skoudridakis and Bourbos, 1990) but to authors knowledge no information are available on the tolerance of the sweet chestnut pathogen toward the fungicide.

Since the 2004 the Department of Plant Protection of the University of Study of Tuscia (Viterbo-Italy) has been elaborating an Integrated Control Protocol (ICP) to manage ink disease of sweet chestnut in the field. The protocol include the use of metalaxyl given as soil drenches around symptomatic trees once in a year.

To determine the possibility to repeat the fungicide treatment for two consecutive years it was essential to investigate the potential insurgence of metalaxyl resistant isolates on *P. cambivora* field isolates recovered before and after those treatments.

As expected based on the literature, the results obtained through this study demonstrate that the resistance to the fungicides is likely to occur after two consecutive treatments with metalaxyl in the field. Even though isolates collected after the first treatment were all sensitive to the fungicide we were unable to affirm that after the first treatment isolates do not display resistance because the number of isolates was very low

On the light of these results the use of metalaxyl for the control of ink disease of sweet chestnut has to be limited to one single application. The immediate consequence of this conclusion is that preventive measures acquire a higher importance within the application of the integrated control protocol which has been suggesting to mitigate the effect of the disease (Vannini *et al*,. 2009; Vettraino *et al*., 2010).

Indeed these type of protocols are often elaborated in view to reduce environmental impacts.

It is expected that this work out-comes guide chestnut-growers toward a correct use of metalaxyl for the control of the disease in the open field avoiding them to use excessively the fungicide which is so often demonstrated to result in extensive development of Phytophthoras resistant isolates.

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CHAPTER IV

Application and validation of an Integrated Control Protocol (ICP) to mitigate ink disease of sweet chestnut

Key words: phosphite; integrated control, *Phytophthora cambivora*

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Abstract

Ink disease of chestnut (*Castanea sativa* Mill.) caused by *Phytophthora cambivora* (Buisman Petri) represents one of the major threats of the cultivation in Italy as in Europe. Its management largely relies on pathogen epidemiology.

Basing on literature reports and pathogen dispersal pathway an Integrated Control Protocol (ICP), including management of superficial water flows, copper sulphate and metalaxyl applications, and potassium phosphite (trunk injections), has been applied in three areas located in Central Italy varying for location, extension, management practices and geomorphology.

Protocol efficacy was evaluated by the mean of disease indicators and through the monitoring of *Phytophthora cambivora* soil inoculum.

The investigation led to an enhancement of disease management guidelines which could be adopted to contain pathogen spread and disease development.

Introduction

Phytophthora causes dieback of native and introduced plants threatening native bushlands such as open forests, woodlands and heathlands, farmlands, nurseries and gardens. It is a major threat to some of European native species (Brasier, 2000).

Several species of *Phytophthora* has been reported to inhabit European forest ecosystems (Brasier, 2000; Jung *et al*., 1996; Jung *et al*., 2000; Vettraino *et al*., 2002) among them *P. cambivora* represents one of the major threat for the sustainability of *Castanea sativa* cultivation (Vannini and Vettraino, 2001; Vettraino *et al*., 2005). The pathogen causes root and collar rot of trees and seedlings in nurseries, plantations and forests. Disease symptoms include crown decline, leaf chlorosis, large roots rot and typical flame shaped necroses of bark tissues at the collar. Under favorable conditions, ink disease pathogens rapidly spread through uncontrolled water flows and transport of contaminated soil particles by humans, animals and vehicles. Thus roads and creeks represent the main pathways of the pathogen dispersal (Vannini *et al*., 2010).

Once an area is infected with *P. cambivora*, eradication is problematic. However, well developed management plans could assist in containing the disease, mitigating the symptoms and preventing further spread to uninfected areas.

Rationally, the control of *Phytophthora* diseases should be based on preventive and curative measures, including management of superficial water flows, the application of fungicides and fungistats, and, eventually, the increase of resistance of threatened trees.

The effect of metalaxyl on the control of ink disease of chestnut was previously reported by Washington (1983) and Vannini and Vettraino (2004). Wicks (1988) demonstrated the efficacy of metalaxyl field applications in drenches around and close to the trunk to control *P. cambivora* crown rot of almond. Metalaxyl efficiently reduces the inoculum density although a relevant risk exists in inducing resistance in *Phytophthora* populations (Erwin and Ribeiro, 1996; Gisi and Cohen, 1996; Parra and Ristaino, 2001; Timmer and Graham, 1998; see also Chapter III of this thesis).

The use of copper based fungicides to control ink disease, particularly Bordeaux mixture, has a long tradition (Vannini and Vettraino, 2004). Sensitivity of *Phytophthora* to copper compounds has been later reported also by Coelho *et al*. (2005).

In the late 1980's, phosphite was demonstrated to be effective in the control of *P. cinnamomi* in agriculture (Cohen and Coffey, 1986) and in native ecosystems using foliar applications (Shearer and Fairman, 1991). Stem injections of phosphite have been previously used to protect chestnut against *Phytophthora* root and collar rot with encouraging results (Lim, 1993). Longevity of action of phosphite for 4-5 years after one injection on *Banksia* and *Eucalyptus marginata* was demonstrated by Shearer *et al*., in 2006. Recently a significant control of ink disease of sweet chestnut, caused by *P. cambivora*, was obtained through the use of both foliar sprays and injection treatments of K- phosphite through a series of field and pot trials (Gentile *et al*., 2009).

These measures have been combined in an Integrated Control Protocol (ICP) and applied in three experimental areas located in Central Italy.

The results of the application of ICP experienced at small and large scale in chestnut groves in Central Italy during the period 2008-2010 will be presented and discussed.

Materials and Methods

The study was carried out in three areas located in Central Italy. The description of each area is reported in table I. The position of chestnut trees in the experimental areas was acquired with a submetric precision, GPS GS20 (Leica Geosystems, Inc. Heerbrugg, Switzerland) and imported on national orthophotos 1:10.000 (Italy, 2000) with the software Arcview ver. 3.1 of the ESRI (Environmental Systems Research Institute, Inc. Redlands, California, USA). Each tree in the experimental areas was tagged and coded.

Disease severity and incidence was evaluated on the base of presence of symptoms typically associated with ink disease. A 5 points visual scale (Vettraino *et al*., 2001) (figure 1) was used: class 0 = healthy trees; class 1 = trees with yellowing of the crown;

class 2 = trees with yellowing and defoliation up to 30% of the crown; class 3 = trees with yellowing and defoliation between 30% and 80%; class 4 = trees completely dead.

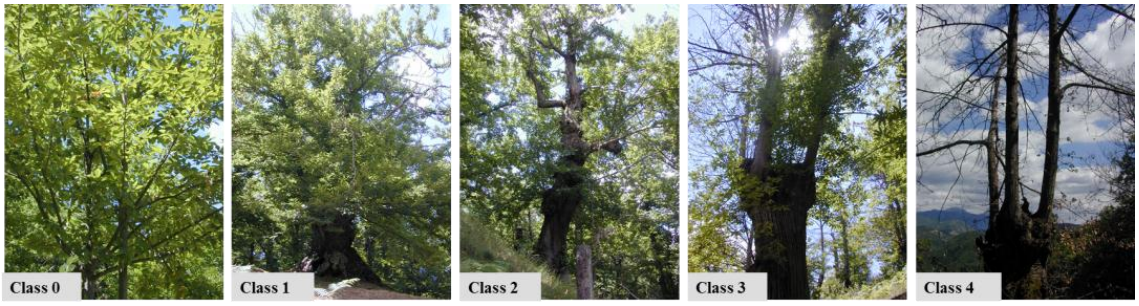

Figure 1. Examples of trees for each class of the scoring scale used to assess severity in the Ink diseased chestnut stands.

ICP included the following measures:

- management of superficial water flows and rural roads by channeling water in a drainage net within the fields (figure 2, 3 and 4);
- treatments of symptomatic plants with metalaxyl RIDOMIL GOLD® Syngenta (~4g metalaxyl/tree) (disease classes 1 to 2) and copper sulphate (~100g/tree) (trees in class 4) in trenches dug around trees (figure 5);
- treatments of natural and artificial drainages and rural roads with copper sulphate (1kg/hl, 10 Kg/ha; 20% of concentration);
- trunk-injections with K-phosphite (Kalex™, AlbaMilagro, Parabiago, Italy) of trees in classes 0 to 2 (15-20 ml/injection; 0.7g/ml) number of injections dependent on the trees size (1 injection each 20-30cm of tree circumference at breast height) (figure 6).

Open channels planning (figure 2) was based on the bioengineering approach with low environmental impact to respect the high naturalness of the areas and because of the lack of sites accessibility and also to minimize the machinery transit within affected areas.

The design solutions consisted in the realization of open channels that could employ as much as possible the natural channels already present in the site. Thus main channels already present within the site were cleaned and re-utilized to constitute the main drainage net. Around these main channels a network of secondary canals with moderate slopes, so as to limit the erosive action of water was developed (figure 3).

A specific discharge of 2 l/s per ha was considered to take in account the possibility of heavy rain periods. Drainages sections were calculated on the base of specific discharge and inter distance between canals.

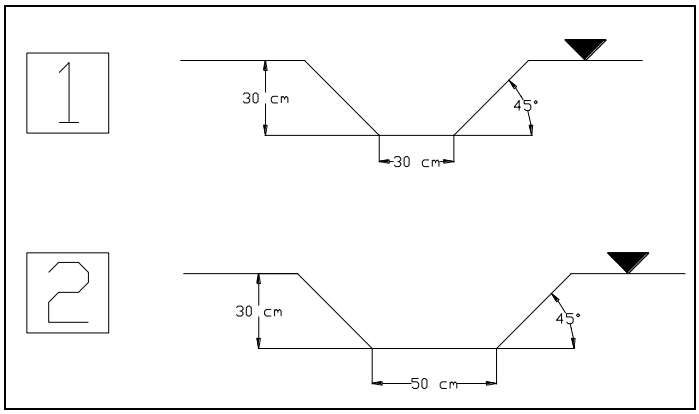

Figure 2. Drainages layout. Sections refer to secondary canals (1) with a width of 30 cm and main canals (2) with a section width of 50 cm.

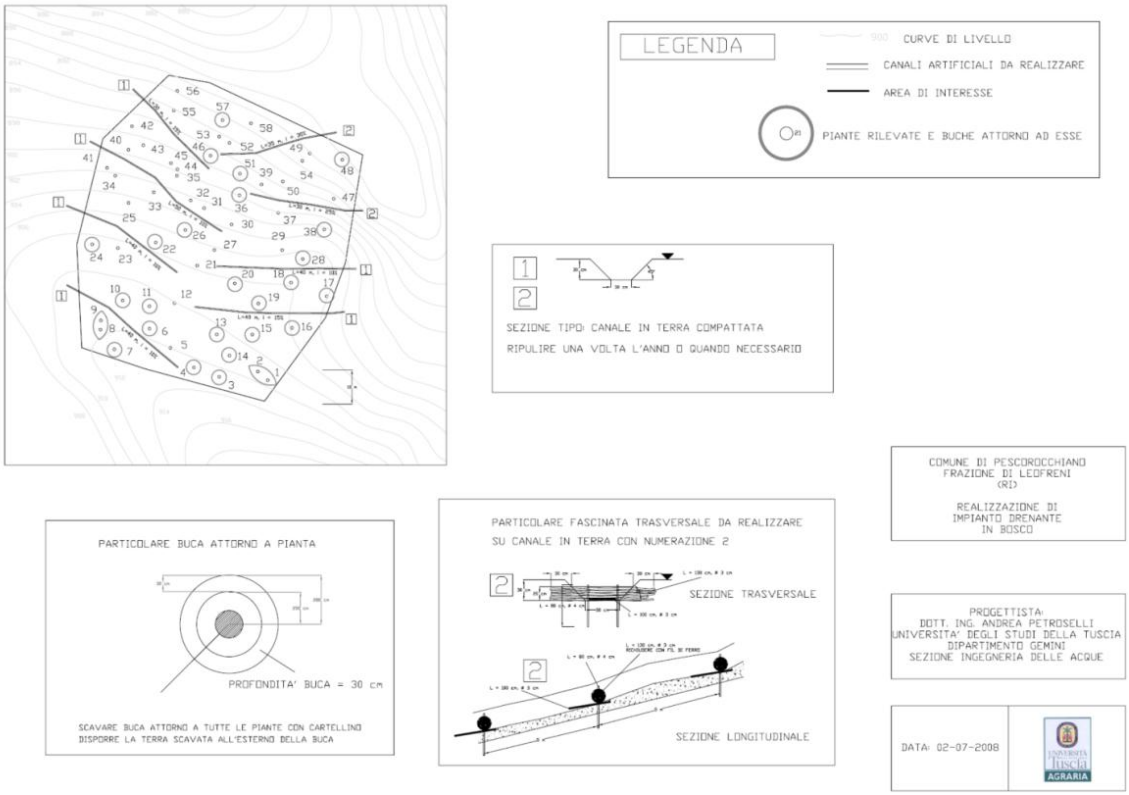

Figure 3. Scheme of the hydrological plan elaborated in collaboration with the GEMINI Department of the University of Tuscia (Viterbo) addressed to manage superficial water channelling the flow in a drainage net within the field. Example refers to the area B in the Rieti Province.

Figure 4. a) Images of the trenches excavated around symptomatic and dead trees; b and c) images of the drainages excavated to allow water flow control within treated plots.

Figure 5. Example of chemical treatment (copper sulphate and metalaxyl) in trenches dug excavated around symptomatic and dead trees in treated areas.

Figure 6. K-Phosphite trunk injections applied at each asymptomatic and symptomatic tree in treated areas. a) Operator during an injections in the area A in Viterbo Province; b) the instrument used to inject trees: Arborjet®.

Additional measures included: restriction of the transit of humans and vehicles on infected groves and along rural roads crossing or bordering the groves, at least during flooding periods; information and training of growers, operators and visitors to adopt hygienic measures to decrease the risk of pathogen dispersal.

An untreated plot was delimited in each chestnut area as control. In areas A and B, ICP protocol was applied in the period spring-autumn 2008. Area B received a second ICP application in 2009. In area C, treatments consisted in K-phosphite injections in August 2008 and July 2009.

In order to quantify the *P. cambivora* inoculum, soil samples were collected from the rizosphere of asymptomatic, symptomatic and dead trees, as well as from roads and creeks before and after each treatment. Baiting technique was chosen as isolation and inoculum quantification procedure (Jung *et al*., 1996; see also Chapter I of this thesis). Pure *Phytophthora* cultures were identified according to their morphological and molecular traits (Erwin and Ribeiro, 1996; Martin and Tooley, 2003).

Efficacy of ICP application has been evaluated by mean of variation of disease severity and incidence (Cardoso *et al*., 2004) and quantification of *P. cambivora* inoculum between treated and control plots, before and after the ICP applications.

The variation of disease incidence and severity was then calculated in the period 2008-2009 and 2009-2010 to evaluate the effect of treatments. To remove the effect of

climatic factors on the expression of crown symptoms, the data were expressed as percentage of the severity of symptoms observed in control plots.

Phosphite residues were assessed in nuts collected from treated and control plots in area B in 2008. Samples within each plot were mixed and divided in 250g each to give 10 lab replicates (treated and untreated). Samples were analysed by ECOTRON (Analytical and technical Services in Modena - Italy). The method adopted for the analysis was PEanions-LCMS (analytic technique LC-MS/MS).

Statistical analysis

Data were elaborated with GraphPad Prism 4.0 (GraphPad Software, San Diego, USA). When Fisher's exact test was performed the percentage of isolation was reported in the graph to make results easier to read.

Results

Description of the experimental areas and the calendar of treatments are showed in tables I and figure 7 respectively. No residues of phosphorous acid were found in the fruits collected after the K-phosphite treatment (table II).

In June 2009 a meeting was organized to inform chestnut growers and operators on the material and methods to adopt to mitigate ink disease within affected areas (figure 8). The same goal have the boards elaborated by the Department of Plant Protection to be placed near the affected areas (figure 9).

Table I. Characteristics of the study areas.

Study Area	Localization	Extensio (hectares	Elevation (m asl)	Slope (%)	Predominant aspect	Number of trees	Kind of soil
A	Carbognano (Viterbo) (Latium Region)	5	410	9.53	S-E	192	Clay-sandy
B	Pescorocchiano (Rieti) (Latium Region)	1.44	840	27.5	S-E	153	Sandy-clay
C	Arola (Ascoli Piceno) (Marche Region)	7.5	980	44.64	S-E	640	Sandy

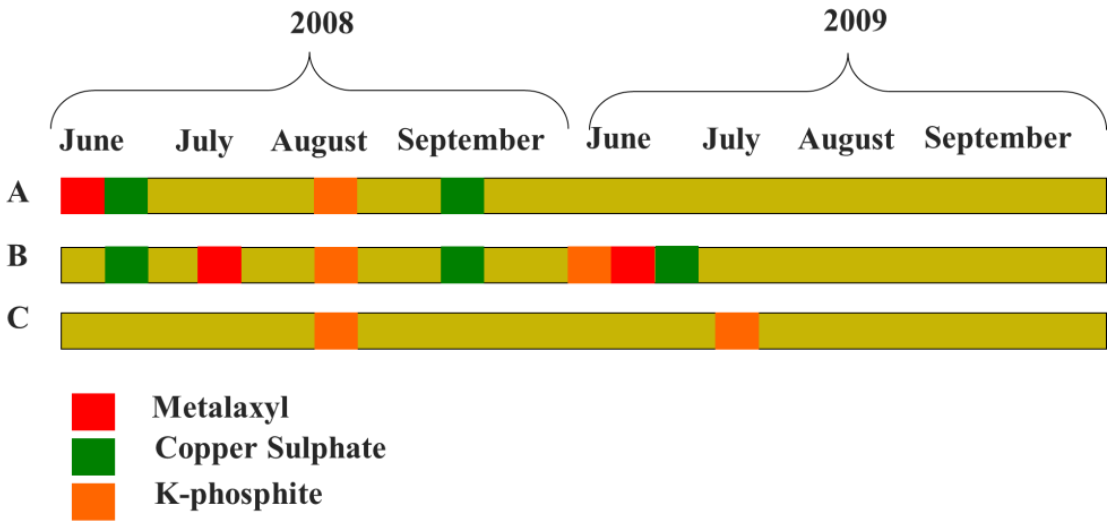

Figure 7. Diagram of the timing of ICP measures applications for each of the experimental study areas located in Central Italy.

Figure 8. Information and training of operators and chestnut growers on materials, methods and results of the ICP.

Figure 9. Boards elaborated by the Department of Plant Protection to alert visitors about measures to reduce the risk of pathogen dispersion in affected areas.

Table II. Analyses results performed on chestnuts collected in treated and untreated plots of area B to assess possible residues on the fruit left from K-phosphite injections.

Ecotron Lab number	Sample	Result*
08T08778	Treated 1	< LQ
08T08779	Treated 2	< LQ
08T08780	Treated 3	< LQ
08T08781	Treated 4	< LQ
08T08782	Treated 5	< LQ
08T08788	Treated 6	< LQ
08T08789	Treated 7	< LQ
08T08790	Treated 8	< LQ
08T08791	Treated 9	< LQ
08T08792	Treated 10	0,131
08T08773	Control 1	< LQ
08T08774	Control 2	< LQ
08T08775	Control 3	< LQ
08T08776	Control 4	< LQ
08T08777	Control 5	< LQ
08T08783	Control 6	< LQ
08T08784	Control 7	< LQ
08T08785	Control 8	< LQ
08T08786	Control 9	< LQ
08T08787	Control 10	< LQ

* Phosphorous Acid content mg/Kg;
LQ=Level Quantity (0.1 mg/Kg)

Effect of ICP application on disease descriptors is showed in figure 10 for area A (Viterbo) and B (Rieti). In treated plots, application of ICP resulted in a decrease of both incidence and severity compared to controls. In particular severity and incidence of

decreased of ca 55 and 49% respectively in area A, and ca 22% and 37% respectively in area B compared to controls.

In site C, where only K-phosphite injections were applied, the variation was 32% for severity and 18% for incidence as measured on treated trees (class 0 to 2) (figure 11).

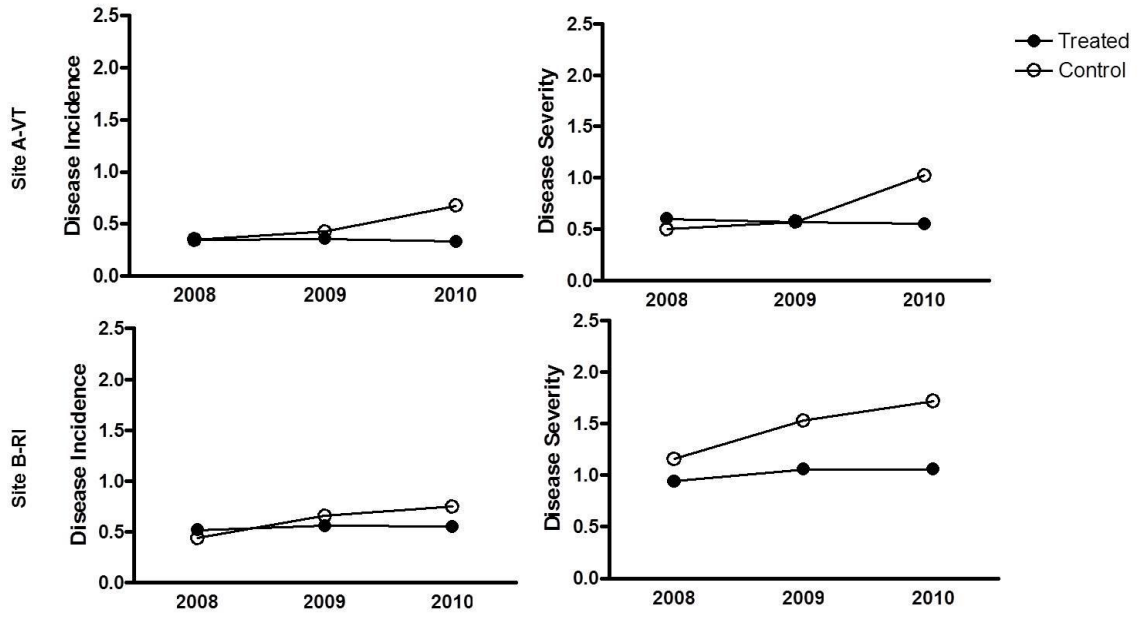

Figure 10. Disease descriptors (incidence and severity) variation for the period 2008-2010 for Area A and B (calculated on all the trees for each plot). Values of 2008, 2009 and 2010 were calculated before, after the first, after the second treatment, respectively. (○) control plot; (●) treated plot.

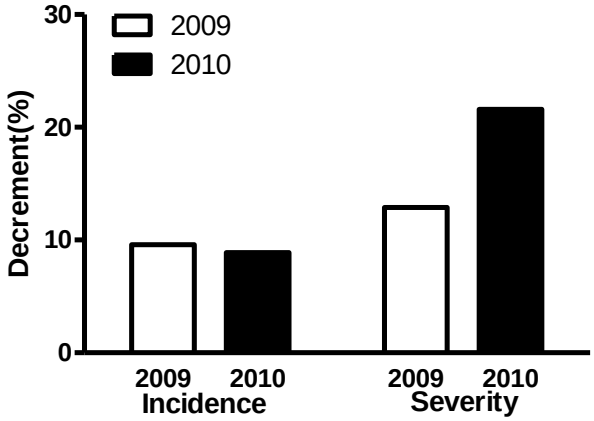

Figure 11. Percent of decrement of incidence and severity of trees injected with K-phosphite compared to control trees in Area C in 2009 and 2010.

The percentage of isolation of the *P. cambivora* varied among areas with the highest value recorded for the Rieti site (B) and the lowest for Viterbo site (A) (see also Chapter II of the thesis).

Before treatments, *P. cambivora* inoculum concentrates mainly in the rhizosphere of symptomatic and dead trees in site B and C. However *P. cambivora* was also recovered from rizosphere of trees in class 0 in all areas (figure 12). No inoculum was recovered from the soil of dead trees in area A because plants were removed together with the whole root apparatus at the moment of their death which happened at least one year before the beginning of the experiment.

P. cambivora recovery varied among sites investigated (see Chapter II of the thesis) and it was found to be influenced by the number of samples assessed at each time (data not shown). In area A the high loam content together with the heavy soil compaction caused by the machinery transit, which characterized the area before the starting of this study, made very hard to consistently isolate the pathogen from soil samples. In area C even if the percentage of isolation gave sometimes good results there was a strong variation between treated and untreated plots. This excessive variation observed in area C was attributed to the significantly smaller number of samples taken in the control area respect to the treated one. Area B was therefore chosen to monitor *P. cambivora* inoculum variation to evaluate ICP applications efficacy because of the highest rate of pathogen isolation and hence the reliability of the results.

Percent of isolation of *P. cambivora* from rizosphere in Area B is showed in figure 13. A positive effect of ICP application on inoculum level was clearly evidenced. Inoculum pattern in the treated plot was well explained by an exponential decay curve (R-square 0.94; not-significant deviation from the model), while regression of data of control plot did not deviate from linearity and even the slope did not differ from 0 (no effect).

Phytophthora spp. inoculum along rural roads, which was monitored since November 2008 in site B, was efficiently decreased after 2009 copper sulphate treatment (figure 14).

There was no effect of K-phosphite on *P. cambivora* isolation from rizosphere of injected trees in Area C compared to control. Linear regression analysis of isolation data calculated in May 2008, 2009 and 2010, indicated not-significant deviation from 0 of the slope for both treated and control trees.

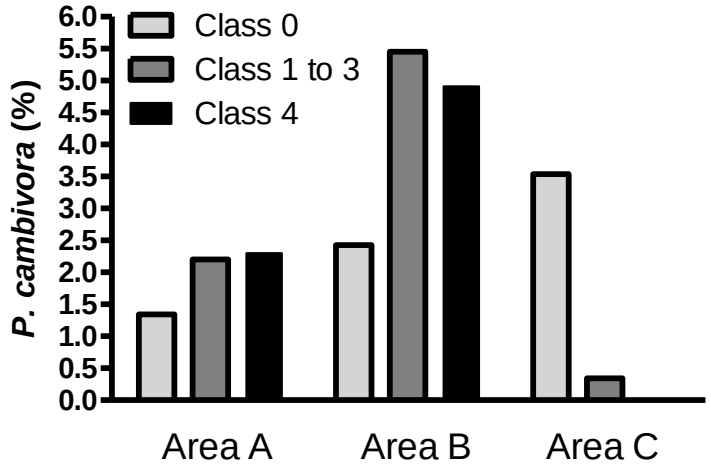

Figure 12. Percent of *P. cambivora* isolation in the rizosphere of trees in class 0 to 4. Results showed by the graph are the mean of the isolations referred to spring-summer 2008, before the beginning of the treatments.

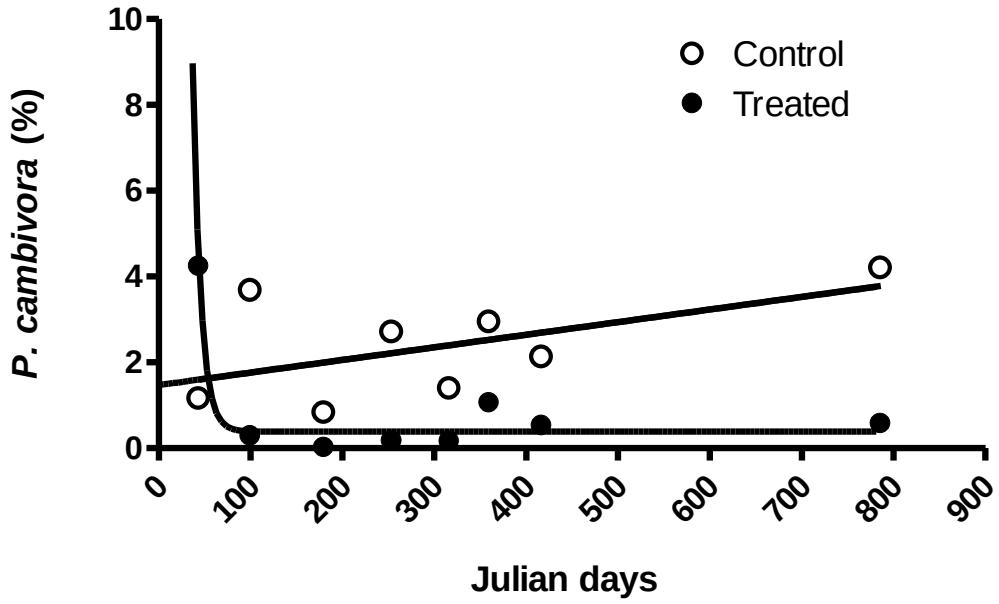

Figure 13. Percent of *P. cambivora* isolation in treated and untreated plots in Area B (Rieti Province) during the ICP application. (○) control plot; (●) treated plot.

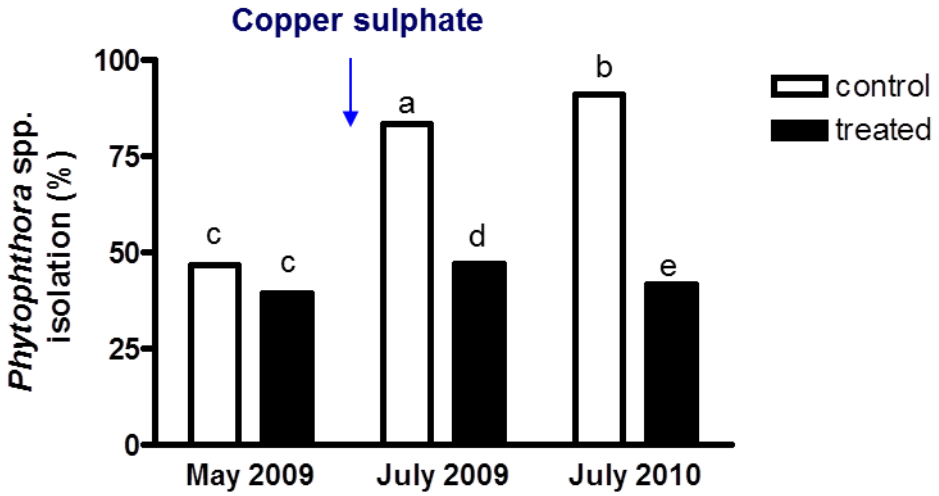

Figure 14. Percent of *Phytophthora* spp. isolation in treated and control roads in Area B (Rieti Province) before (May 2009) and after (July 2009) copper sulphate treatment made on June 2009. Different letters above columns indicate significant differences ($P<0.0001$) between groups of values (Fisher's exact test).

Discussion and conclusions

This study was undertaken to elaborate useful management guidelines to mitigate the effect of ink disease of sweet chestnut through the application of integrated control measures (ICP) within affected orchards.

Results highlighted a positive effect of ICP application to control ink disease in chestnut stands in Central Italy. A single application of the ICP protocol was effective in slowing down the disease progression and even in reducing the level of inoculum in the soil.

The greatest effect was achieved in the orchard characterized by the lowest disease severity as assessed at the beginning of the experimentation supporting the hypothesis of Gentile *et al*. (2009) that efficacy of treatments could be negatively correlated with the initial disease severity. An appreciable reduction in disease progression was also obtained in Area C where trees received only K-phosphite in absence of any water flow management and chemical treatment on trees, roads and creeks. Similar results were reported for the same and other *Phytophthora*-host binomial by other authors (Garbelotto *et al*., 2006; Gentile *et al*., 2009; Jung *et al*., 2010; Solla *et al*., 2009). However, this study represents the first record of effect of K-phospite alone on mature chestnut trees. In fact the beneficial effect of K-phosphite treatments of ink diseased chestnut plants has been recently reported by Gentile *et al*. (2009) in greenhouse experiments and in field on young trees. K-phosphite concentration applied in the present study was the same reported by Gentile *et al*., 2009. K-phosphite application to

trees was not able alone to reduce the inoculum in the soil along the tested period. Thus the combination of copper and metalaxyl application together with K-phosphite injection seems to be the better choice to mitigate the effect of ink disease by controlling both inoculum level in soil and protect trees from pathogen colonization of tissue.

The beneficial effect of copper has been highlighted in roads, where it was very efficient in reducing inoculum vitality. The use of copper can be considered essential in controlling inoculum transport along preferential pathways.

This study also report for the first time that K- phosphite could be considered safe for human health as no harmful residues were found in the fruits collected two months after trunk injections.

The use of metalaxyl should be considered carefully. The ascertained insurgence of resistant strains within field isolates of *P. cambivora* after two series of field treatments with metalaxyl (see Chapter III of this thesis) would recommend to limit its use at one single application. This is in accordance with what reported by Pegg and Whiley, (1987) who talked about "a dramatic deterioration in tree health following three years of exclusive and continuous use" of metalaxyl for the control of *P. cinnamomi* root rot of avocado.

Copper is not inducing resistance in microorganisms. However a growing awareness is put in the use of this molecule do to its accumulation in soil (Han *et al*., 2000; Besnard *et al*., 2001) and the resulting toxicity against other soil microorganisms alike important micobiont such as vesicular arbuscular mycorrhizal fungi (Graham *et al*., 1986). A single copper treatments is then suggested in spring before seasonal rain and increase of soil temperature.

Best period for K-phosphite trunk injections for chestnut is immediately after flushing that at Italian latitudes coincides with May beginning of June .

In view of the accumulation of *Phytophthora* inoculum along rural roads and creeks the importance of preventive measures adoption within the ICP is further strengthen. Unfortunately this is the part of the protocol more difficult to reach because correlated to people's awareness which not often was found to characterize growers and field operators to whom those measures are addressed.

Still the cost of ICP application in terms of person work and material results quiet high. In particular the management of superficial water flow and roads maintenance are cost

effective even for the heterogeneity of the landscape that characterizes chestnut groves in Italy.

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CHAPTER V

Summary

The main objective of this thesis was to evaluate and validate an Integrated Control Protocol (ICP) to mitigate ink disease in chestnut groves.

The protocol was developed by integrating physical and chemical measures already reported in scientific literature as efficient to control Oomycota.

Validation of ICP application was based on, alleviation of crown symptoms expressed as severity and incidence, and reduction of soilborne Phytophthoras inoculum.

While crown symptoms were assessed with a standard visual scale, living inoculum level was evaluated with an optimized baiting protocol proved to be efficient and sensitive to bait the complex of species commonly present in chestnut soils.

An additional issue was related to the sampling strategy that it is likely dependent on the knowledge of the preferred localization of Phytophthoras over a heterogeneous landscape. Thus extensive surveys were conducted to determine the complex of inhabiting Phytophthoras in different landscape traits as like as creeks, roads and trees rhizosphere. In order to achieve this objective, major efforts have been addressed to optimize methodologies as diagnostic tools to detect living inoculum of *Phyhtophthora*.

These sampling activities allowed to advance the knowledge on the complex of Phytophthoras inhabiting chestnut soil, producing new records on chestnut for Italy and Europe. Furthermore the risk for chestnut posed by an additional invasive widespread species, *P. megasperma*, was evidenced. However these surveys also confirmed the presence of Phytophthoras inoculum associated to roads and creeks.

Studies carried out on *P. cambivora* isolates recovered before and after metalaxyl treatments revealed an high risk of induced resistance to this chemical. In fact, as already reported for many other Phytophthoras, this study confirmed that continuous metalaxyl applications could enhance the risk to induce resistance in pathogen field isolates restricting, consequently, the possibility to use this fungicide.

Thus, after two years of study, the original ICP protocol could be validated with some modification:

1. water flow management: very important to channel superficial running water in drenches along roads and in natural creeks;
2. copper treatments: very important in artificial channels dug along roads as well as in the net of natural and artificial drainages that regulate the flow of superficial water. Also important to reduce inoculum from rizosphere of dead trees;

3. metalaxyl treatments: important to reduce inoculum in the rizosphere of infected trees. However insurgence of resistant isolates imposes a max of one treatment. Copper might be used instead;
4. K-phosphite treatments: very important to increase resistance of trees but not useful to reduce the inoculum in the soil. Thus, K-phosphite alone might stop disease effect but pathogen spread.

As a conclusive remark, application of ICP as the combination of different control and hygienic measures can mitigate the effect of ink disease in orchards and slow down the spread of the inoculum. Our experimental data showed that beneficial effect are still evident after one year from the last application. However the respect of additional hygienic measures as like as to close the transit in infected areas and to disinfect shoes, tires of people or vehicle transiting through infected areas is believed to be essential to increase the chance of success.

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