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In early 2000, unusual mortality of a native North American tree, golden chinquapin, was reported by the USDA-Forest Service. Dying trees exhibited girdling cankers in the inner bark of the lower bole, branch flagging and defoliation. Isolations from necrotic tissues and soil associated with diseased or killed trees yielded *Phytophthora cambivora,* a pathogen that is known to infect and kill chestnut species in Europe and in the United States.

Morphological, physiological and molecular testing confirmed the identity of isolates recovered as *P. cambivora.* Pairing tests showed that both mating types, A1 and A2, of this species were present in forest soil in Oregon.

Pathogenicity tests were conducted to confirm the susceptibility of golden chinquapin to *P. cambivora.* Two inoculation trials were conducted: 1) Trees were wound inoculated with mycelial plugs of *P. cambivora*; after 35 days necrotic tissues were formed in the inner bark of all the inoculated trees, as seen in natural infections. 2) Seedlings were inoculated with a zoospore suspension of *P. cambivora*; after 38 days all inoculated seedlings were killed, the roots were rotted and the inner bark of lower stems was necrotic. *Phytophthora cambivora* was re-isolated from necrotic tissues in both trials, completing Koch's postulates.

Susceptibility of Golden Chinquapin (*Chrysolepis chrysophylla*) to *Phytophthora cambivora*

by

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Angel Luis Saavedra, Author

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Chapter 1

Introduction

1.1 The problem

A new canker disease was reported in recent years (Goheen pers. comm.) causing mortality of golden chinquapin trees (*Chrysolepis chrysophylla* (Dougl.) Hjelmqvist) growing in the southwestern Oregon Cascade Range in the Butte Falls Ranger District of the Rogue River-Siskiyou National Forest. The cankers on the boles of golden chinquapin trees look similar to those that are caused by species of *Phytophthora* (Oomycetes) on other species of North American forest trees (Tainter et al. 2000; Rizzo et al. 2002; Betlejewski et al. 2003). The cankers in the inner bark were reddish-orange in color extending upward from infected roots. Leaf flagging was a commonly observed symptom of infected trees, especially on the lower branches. The leaves of affected trees turned from their normal green to a bright red, almost like the tree is on fire, and eventually were dropped. Preliminary surveys showed that most of the symptomatic or dead trees were located near roads, although in some cases individual trees located further away from roads were symptomatic.

This area of southwestern Oregon where symptomatic chinquapins have been observed is approximately 28 miles east of Medford, OR on State Highway 140 and at

approximately 1 300m above sea level. The site's geographic co-ordinates are 42° 23' north latitude and 122° 22' west longitude. In this area, golden chinquapin is associated with a dominant coniferous forest that includes Douglas-fir (*Pseudotsuga menziesii*), western white pine (*Pinus monticola*), western hemlock (*Tsuga heterophylla*), white fir (*Abies concolor*), and Pacific yew (*Taxus brevifolia*). Other common plant species associated with this forest community include Pacific madrone (*Arbutus menziesii*), California (western) hazel (*Corylus cornuta* var. *californica*), Pacific dogwood (*Cornus nuttallii*), Pacific rhododendron (*Rhododendron macrophyllum*), and Oregon grape (*Berberis nervosa*).

In order to determine a possible causal agent of this new canker disease, samples of bark from the actively growing canker margin were collected and plated in *Phytophthora* selective medium (Hansen and Goheen, pers. comm.). Isolates of a *Phytophthora* species were readily obtained from the cankers, suggesting that this organism could be the causal agent of the disease. To further investigate the distribution of this organism in the area, soils samples were collected from the bases of dying trees and baited using standard techniques as described by Erwin and Ribeiro (1996). A *Phytophthora* species was consistently recovered supporting suspicions that a soilborne *Phytophthora* was infecting golden chinquapin through the roots and causing the lethal cankers.

Because isolates from soil and from cankers on dying golden chinquapin were similar in morphology to *P. cambivora*, and also because this species is well known in Europe as the causal agent of root rot and collar rot on commercially and ecologically important hardwood species such as *Juglans regia, Fagus sylvatica* and *Castanea sativa* (Delatour,

2001; Jung et al. 2003; Vettraino et al. 2003), it was hypothesized that *P. cambivora* was the causal agent of this new canker disease of golden chinquapin.

The objectives of this study were to identify the isolates recovered from dying chinquapin and surrounding soil by morphological and molecular comparison with known *Phytophthora* species, and to determine their association with this new canker disease by satisfying Koch's postulates. Pathogenicity tests on golden chinquapin seedlings under greenhouse conditions as well as inoculation in bark of healthy chinquapin trees in the field were completed.

1.2 *Phytophthora cambivora* (Petri) Buisman

1.2a. Description of *P. cambivora*

Petri (1917) in his research on root rot of chestnut in Europe described this species for the first time under the name of *Blepharospora cambivora* Petri. Ten years later, Christine Buisman (1927), as part of her thesis, argued that the characters on which the genus *Blepharospora* was founded could not be considered distinct from the genus *Phytophthora* and so in 1927 this species was synonymized under *Phytophthora*.

Members of the genus *Phytophthora* absorb nutrients from the environment, have filamentous mycelia, and reproduce by spores. They share these features with true fungi, however, the genus *Phytophthora* possesses zoospores with two flagellae and the absorptive hyphae are diploid, among several other important differences. Current taxonomic classifications place this genus under the Kingdom Chromista, within the family Pythiaceae of the order Peronosporales (Cavalier-Smith, 1986; Kendrick, 2000).

Under this classification, the genus *Phytophthora* is more closely related to brown algae than true fungi (Eumycota).

The sporangia of *P. cambivora* are non-caduceus and non-papillate and their antheridia are amphigynous. On the basis of these morphological features, Waterhouse (1963) classified *Phytophthora cambivora* under group VI which includes another important plant pathogen, *Phytophthora cinnamomi.* Morphologically, these two species differ because *Phytophthora cambivora* produces bullate oogonia and no chlamydospores. It should be noted that the Waterhouse morphological grouping of the genus *Phytophthora* does not necessarily represent phylogenetic relationships, as was demonstrated by Cooke et al. (2000). Under the latter phylogenetic scheme, *Phytophthora cambivora* was placed in the same clade as *Phytophthora cinnamomi.*

Interspecific hybridization is a concept that has been proposed to explain pathogenicity pattern changes on the genus *Phytophthora* (Brasier, 2000; May et al. 2003). It hypothesizes that progeny resulting from interspecific hybridization could exhibit reduced aggressiveness toward the parental hosts or host ranges that differ from those of parental species. The latter is the case with a recently described new species of *Phytophthora* (*P. alni*) in Europe affecting trees of the genus *Alnus* (Brasier et al. 2004). The parent species are apparently *P. cambivora* and a species of *Phytophthora* related to *P. fragariae.* It is also important to note that *P. cambivora* is a heterothallic species, meaning that it requires a compatible opposite mating type to complete sexual reproduction.

1.2b. Infection and spread biology

Most members of the genus *Phytophthora* are plant pathogens and *P. cambivora* is not an exception. It is known that *P. cambivora* is a soil born pathogen and the lack of chlamydospores suggests that the primary propagules of this pathogen consist of swimming zoospores. In areas where compatible mating types are found and oospores are formed, it can be inferred that these structures also can constitute a source of inoculum. It can also be inferred that propagules of *P. cambivora* can be carried around in the soil by many vectors including human, and that it may be distributed in from infested areas to non-infested ones. Infection is presumed to occur through the roots of trees, since it has been observed that the biomass of roots growing in soil free of *Phytophthora* inoculum is greater than the mass of roots growing in infested soil (e.g. *Quercus* spp) (Delatour, 2001) and the pathogen causes root rot on *Prunus* spp (Mircetich and Matheron, 1976) and on English walnut (Vettraino et al. 2003). The results of the latter study showed that *P. cambivora*, as well as other species of *Phytophthora*, significantly reduced the biomass of roots of walnut seedlings growing in infested soil with *Phytophthora* compared to uninoculated control roots. This suggests that this pathogen's primary way of infecting its host is through the roots.

Whether *P. cambivora* can penetrate host tissues by other means than through their roots is not known. Artificial inoculations of recently cut logs of conifer and hardwood species (Hansen and Sutton, 2002) as well as seedling stem inoculations show that, given the proper conditions, *P. cambivora* can successfully infect trees through wounds (Delatour, 2001 and personal experience that will be discussed in Chapter 2).

Another mechanism of spread is by means of infected rootstocks as was documented by Jeffers and Aldwinckle (1988). In this study, unbudded apple rootstocks from shipments received by local nurseries from the major rootstock suppliers were sampled before they were planted. Bioassay techniques included washing the rootstocks and plating the rhizosphere soil on PAR (pimaricin, ampicilin and rifampicin) and PARH (PAR plus Hymexazol) selective media, as well as baiting the soil using apple cotyledons as baits. They recovered *Phytophthora cambivora* 111 times from 153 roots of unbudded clonal apple rootstocks. They also reported that most recovered isolates of *P. cambivora* were mating type A1 (92 isolates) and 3 isolates of mating type A2. This suggests that opportunities for intraspecific sexual reproduction, and hence oospores as a source of host infection, were reduced.

1.2c. Distribution of *Phytophthora cambivora*

According to the Commonwealth Mycological Institute (CMI) Description of Pathogenic Fungi and Bacteria No. 112 (Waterhouse and Waterston, 1966), *Phytophthora cambivora* has a worldwide distribution (Table 1).

Table 1. Geographical distribution of *Phytophthora cambivora*

Continent	Countries
Africa	Mauritius
Asia	India
Australia & Oceania	New Zealand
Europe	Azores, France, Great Britain, Italy, Poland, Portugal, Spain, Switzerland, Turkey and Yugoslavia
North America	Canada and the United States

Since this publication, *P. cambivora* has been reported from many other countries including South Africa, Japan, Australia and Scotland and is associated with diseases of endemic trees as well as ornamental crops (Gerretson-Cornell 1978; Erwin and Ribeiro, 1996). Several researchers during the past few years have studied the involvement of *Phytophthora* species with a complex disease known as "oak decline" in Europe. Vettraino et al. (2002) reported recovering *P. cambivora* and eleven other *Phytophthora* species, from sites associated with oak decline syndrome in the southern and central part of Italy and with chestnut in the Italian northern region. Jung et al. (2000) investigating the distribution of *Phytophthora* species from different oak stands in Bavaria, Germany recovered *P. cambivora* from soil. Researchers from other countries such as England and Sweden investigating the matter have recovered *P. cambivora* from oak stands (Brasier and Jung, 2001; Jönsson et al. 2003 respectively).

Phytophthora cambivora is also associated with another important disease known as "ink disease" in Europe and in the USA. Studying the distribution of the disease in chestnut stands in Europe, Vettraino et al. (2005) reported recovering isolates of *P. cambivora* from chestnut in Greece, Italy and France.

The first report of the occurrence of *P. cambivora* in the United States was by Pirone (1940). He reported mortality of hundreds of maple trees in New Jersey due to a basal canker associated with the *P. cambivora* group. Other studies have reported the occurrence of *P. cambivora* in California and New York, always associated with diseases affecting fruit orchards (Mircetich et al. 1974; Mircetich and Matheron, 1976; Jeffers and Aldwinckle, 1988). In the Pacific Northwest, *P. cambivora* has been isolated from dead

roots and girdling stem cankers on Noble fir in Christmas tree plantations (Hamm and Hansen, 1987; Chastagner et al. 1995).

This pathogen has also been reported from New Guinea (Ash, 1988). In this study, searching for explanations of the observed dieback of *Nothofagus*, *P. cambivora* along with *P. cinnamomi* Rands were isolated from soil samples in *Nothofagus* forest in New Guinea. Both *Phytophthora* species were isolated from healthy and declining stands and no conclusion could be inferred regarding pathogenicity on *Nothofagus*.

More recently, Greslebin et al. (2005) isolated *P. cambivora* from soil associated with *Austrocedrus chilensis* forest in Patagonia, Argentina. It was recovered from soil in only one of eleven sites of the study; whether this pathogen is associated with the observed decline of *A. chilensis* is still not known.

I n summary, *P. cambivora* has a worldwide distribution and because it has been found associated with healthy, declining, and dead stands it is likely that this pathogen occurs in many other places yet to be described.

1.2d. *P. cambivora* host range

Erwin and Ribeiro (1996) reported a list of hosts susceptible to *P. cambivora* that includes more than 30 species in 12 families of plants. Depending upon the host, this pathogen causes diseases that include wilt, blight, collar rot, root rot and trunk canker. Most of the hosts included in the list are of economic and ecological importance; among others are species of the genera *Acer, Castanea, Casuarina, Fagus, Juglans, Malus, Nothofagus, Persea, Prunus, Rhododendron, Rubus and Ulmus*. The list also includes

members of the Asteraceae family that are cultivated for medicinal purposes such as *Chrysanthemum cinerariafolium* and nursery plants like species of the genus *Senecio*.

Phytophthora cambivora has been isolated from a number of species of conifers grown for Christmas trees in the Pacific Northwest of the United States. Hamm and Hansen (1987) reported isolating *P. cambivora* from rotted roots and stem cankers on 2-5 year-old Noble fir but pathogenicity was not confirmed at the time. Chastagner et al. (1990a) and Chastagner et al. (1995) also reported isolating this pathogen, among other species of *Phytophthora*, from stem cankers and root rot of symptomatic Noble fir trees in plantations in Oregon and Washington but did not test whether *P. cambivora* could cause root rot and stem canker and consequently mortality on Noble fir. However, Chastagner et al. (1990b) reported a pathogenicity test in which stems of field grown Noble fir were inoculated with species of *Phytophthora,* including *P. cambivora,* in order to study their ability to cause canker. The results indicated that *P. cambivora* was among the most virulent species of *Phytophthora* causing stem canker in Noble fir. In this same report, eleven species of *Abies* were grown in soil infested with several species of *Phytophthora* in order to determine susceptibility of these hosts to these pathogens. *Phytophthora cambivora* was found to be virulent to susceptible species of *Abies* but *P. cinnamomi* caused the most mortality.

1.2e. Important diseases caused by *P. cambivora*

Among the diseases caused by *P. cambivora,* the ink disease of chestnut in Europe and in the United States is considered to be the better known and studied disease (Erwin and Ribeiro, 1996; Vettraino et al. 1999; Bourbos and Metzidakis, 2000). This disease is

characterized by trunk and root rot and consequently tree mortality. Another feature of this disease is the occasional exudation of inky fluid from dying or dead bark at the base of the trunk. Little is known about how this pathogen is moved around in nature. However, it is likely that it is carried in mud by humans and animals as noted by Vettraino et al. (1999). In addition, this report states that in sweet chestnut plantations in Italy, the disease commonly starts from trees along roads and trails. This pattern has also been observed on cankered golden chinquapin trees, as will be discussed in chapter 2 of this dissertation.

Due to the economic importance of sweet chestnut (estimated to have an import wholesale value approaching $20 million in Italy alone) and the fact that great concern is apparent in Europe regarding a potential new outbreak, a considerable amount of funding for research is being put into place in search of a better understanding of this disease. It has been estimated that annual consumption of chestnut per capita is around 2 lb. in China, 1 lb. per capita in Europe and less than half a pound per capita in the U.S. Another reason that chestnut ink disease receives so much attention is to try and avoid ecological catastrophes like the one caused by *Cryphonectria parasitica* on American chestnut early in the 20th century in the United States.

Other diseases of economic importance caused by *P. cambivora* are crown and root rot of many fruit trees such as plums, apples, apricot, peaches and cherry among others (Mircetich and Matheron, 1976; Jeffers and Aldwinckle, 1988; Erwin and Ribeiro 1996). Unfortunately, above ground symptoms of *Phytophthora* infected trees resemble those caused by other root rot pathogens, so direct isolation from infected plant tissues is necessary to determine whether an infection has been caused by *P. cambivora*.

Phytophthora cambivora, as well as other species of *Phytophthora,* has been associated with oak decline in Central Europe (Jung et al. 2000). In this study, *Phytophthora cambivora* was among the most frequently recovered species, but only from soil samples. Whether there is a correlation between the occurrences of *P. cambivora* in the soil and the severity of oak decline is still undetermined. It was noted in this study that characteristics of the site (soil pH, drainage, texture) were strongly related to the presence of *Phytophthora* species.

1.3. *Chrysolepis chrysophylla*

Golden chinquapin *(Chrysolepis chrysophylla),* also known as giant chinquapin, is a hardwood species that grows in a landscape dominated by coniferous forests. In Oregon and northern California, golden chinquapin grows in tree and shrub forms in a variety of habitats but it is rarely a dominant component of any stand. The tree form can be found growing as stem clumps or as solitary trees. Toward its southern range, trees commonly grow as tall as 31 m and reach circumference of 120 cm in diameter (Jensen and Ross, 1995). Golden chinquapin is an evergreen species; its leaves are arranged in an alternate pattern on the branches and they are coated underneath with golden yellow leaf-hair, hence its common name. Even though its wood posseses excellent qualities for furniture making and for hardwood plywood, this species is considered in many areas of its natural range to be a competitor of commercial species.

1.3a. Distribution-range

Mc Kee (1990) reported that the natural range of golden chinquapin extends from San Luis Obispo County in California, to Mason County in western Washington. He noted that in California, golden chinquapin grows primarily in the Coast Ranges, but there is a disjunct population in El Dorado County in the Sierra Nevada. In Oregon, it is found in the Coast Ranges as far north as Benton County, and throughout the Cascade Range as far north as Marion County (pers. observations). In Washington, golden chinquapin is represented by two disjunct populations in Mason and Skamania Counties (Kruckeberg, 1980).

Shrub forms of the species are found throughout its range. The tree form is primarily distributed from Lane County, OR, south to Marin County, CA. It is found from near sea level in the Coast Ranges of Oregon and California to over 1525 m in elevation in the Cascades. Although golden chinquapin is generally thought of as a mid- to low-elevation species, the shrub form can be found along the crest of the Cascade Range in Oregon from 1525 to 1830 m.

1.3b. Taxonomy

The species *Chrysolepis chrysophylla* (Dougl. ex Hook.) Hjelmqvist is classified under the botanical family of Fagaceae. Members of this family are characterized as catkin-bearing trees and shrubs with the fruit, an acorn, at least partially enclosed by a cupule (Smith, 1977).

Golden chinquapin staminate flowers are creamy white and arranged in catkin-like inflorescences. The flower odors that are perceived during the summer as it blooms are

distinctive of this genus. The female flowers are borne within an involucre at the base of male flowers or can be located alone along the stem. The nuts, which mature in the fall of the second growing season, are enclosed in a spiny burr that is unique for this genus (Manos et al. 2001).

The genus *Chrysolepis* includes only two species and both are native to the western part of the United States; *C. chrysophylla* and *C. sempervirens*. The separation of this genus from the previously known genus *Castanopsis* occurred in 1948 on the basis of the cupule structure (Hjelmqvist, 1948). The genus *Castanopsis* was reserved for the subtropical and tropical species that occurred in Asia.

Finally, naturally occurring and cultivated species of "chinquapin" in the SE United States are members of the genus *Castanea*. According to Li et al. (2004), *Castanea*, *Castanopsis* and *Chrysolepis* are distinct but closely related genera.

1.3c. Diseases affecting golden chinquapin

There are few reports of diseases or insects that affect the growth or survival of golden chinquapin. (Hepting, 1971). However, it is known that it is very susceptible to a number of root rot pathogens, heart rots, and a few common foliar fungi (Farr et al. 1989). Among these organisms heart rot caused by *Phellinus igniarius* is known to be damaging to golden chinquapin, as well as root rot caused by *Armillaria* species. During the current study infection by *Armillaria* on golden chinquapin trees was observed at different sites in its natural range. However, no attempt was made to identify the species of *Armillaria*.

There are very few insect pests on golden chinquapin. Seed-feeding insects are the most commonly reported. These insects could play a significant role in reducing golden chinquapin trees' regeneration capacity depending on the percentage of infested fruits.

Dryocosmus castanopsidis, a cynipid wasp attacks the staminate flowers causing spherical golden-yellow to brown galls (Goheen pers. comm.). The amount of infested flowers also varied by region and from tree to tree. No study has looked into its effects on golden chinquapin reproduction.

1.3d. Ecological role and uses

Although, golden chinquapin has a light brown and fine grained wood, it has little commercial value because trees of timber size are not abundant at any one place (Brockman, 1958; Jensen and Ross, 1995). It is known that wood of golden chinquapin was used for making agricultural tools and several other items that required strong wood (Uphof, 1959).

Ecologically, golden chinquapin trees are considered of importance for wildlife. Its nuts are nutritious and eaten by a variety of birds and small mammals. The restriction is the availability of fruits, since they are produced at irregular intervals and in low numbers (Mc Kee, 1990).

Golden chinquapin shrubs are rarely browsed by livestock, but certain shrubby ecotypes are of moderate importance as mule deer browse in portions of California. However, golden chinquapin bushes are browsed only when the preferred browsing species is unavailable due to overgrazing or other reasons (Hubbard, 1974). Historically, golden

chinquapin nuts were roasted and eaten by indigenous people throughout the Coast Ranges of northern California and southwestern Oregon (Uphof, 1959).

1.4. Thesis objectives

I want to establish the identity of the *Phytophthora* isolates recovered from dying chinquapin trees and surrounding soil and to determine their association with this new bole canker on golden chinquapin by completing Koch's postulates. To accomplish these objectives, the following activities were undertaken:

1). Three Oregon isolates resembling *P. cambivora* were characterized in terms of their mating type, ITS rDNA sequence, and morphological and physiological features.

2). Field inoculation of live trees with the recovered isolates resembling *P. cambivora* were made to observe necrotic lesion formation, possible differences in aggressiveness among them, and their re-isolation to complete Koch's rules.

3). Seedlings of golden chinquapin were inoculated under greenhouse conditions with the recovered isolates resembling *P. cambivora* to demonstrate susceptibility of seedlings to zoospores of this pathogen. Mortality was measured, further satisfying Koch's Postulates.

Our hypothesis is that the recovered isolates belong to *P. cambivora* and that this species is the causal agent of this new canker disease reported in boles of golden chinquapin in Oregon.

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Identification and pathogenicity of a *Phytophthora* species isolated from dying Golden Chinquapin trees and Oregon forest soil

Chapter 2

2.1 Introduction

Golden chinquapin (*Chrysolepis chrysophylla* (Dougl) Hjelqvist) is an evergreen tree species native to western North America. It grows in a variety of woody plant communities but it is most commonly found growing in mixed stands of conifers, especially Douglas-fir, since golden chinquapin rarely occurs in pure stands. Its native range extends from California (San Luis Obispo County) to Washington (Mason County). It can be found growing in both the California and Oregon Coast Ranges and in the Oregon Cascade Range; however in Washington only two isolated populations have been found (Mason and Skamania Counties) in several surveys conducted in the State (Brockman, 1958; Kruckeberg, 1980). Golden chinquapin can be found growing from mid to low elevation ranges but it also can be seen above 1 500 m, growing mainly in its shrub form.

This cone-triangular shaped tree that commonly grows up to 30 m tall and 120 cm in diameter, stands out within the mixed forest where it grows due to its distinctive oval to lanceolate, alternate leaves. When trembled by the wind their beautiful underneath golden scale is revealed, which gives the common name of "golden chinquapin". In addition, a strong musky odor that its flowers emit during late spring and early summer reveals the presence of golden chinquapin in mixed stands.

Golden chinquapin fruits are triangular nuts enclosed inside sharp spiny burrs resembling those of chestnuts (*Castanea* spp). The nuts are edible, and used by wildlife. Botanist David Douglas (1799-1834) (Cited by Scheffer, 1961) wrote that after he shot a band-tailed pigeon and examined the bird's stomach contents he found chinquapin nuts.

The market for golden chinquapin wood is very small, even though the wood is considered to have excellent qualities for furniture and for construction lumber (McKee, 1990). Use is limited because the wood is very difficult to cure, very few mills are equipped to process it, and pure stands rarely occur in nature. Other species of "chinquapin" (*Castanea* spp.) in the southeastern United States are cultivated for their sweet edible nuts, and seedlings of these species, especially *Castanea pumila* are available in nurseries (Payne et al. 1993). Unfortunately, this is not the case for golden chinquapin, even though the nuts are edible and a small market exists for the wood. Golden chinquapin has a poor rate of seed germination, ranging from 14 to 53 percent (Hubbard, 1974).

There are not many reports of diseases or insects that might affect the growth or survival of golden chinquapin (Farr et al. 1989). However, it is susceptible to root rot caused by *Armillaria* species, flower galls caused by *Dryocosmus captanoisidis* (Russo, 1979 and personal observations) and defoliation caused by an unidentified ascomycete fungus reported by Kruckeberg (1980) in Washington. Unlike its close relatives, *Castanea* spp. in Europe and in North America, golden chinquapin is not susceptible to *Cryphonectria parasitica* (Anagnostakis, 1987; Barnard, 2000).

Observations of increasing mortality of golden chinquapin trees in southwestern Oregon (Don Goheen, personal communication) were brought to the attention of the

scientific community in 2001 (Hansen, 2001). The problem was described as a new lethal canker disease of golden chinquapin characterized by girdling basal cankers extending upward from infected main roots. The leaves of affected trees turned bright red and later dropped, with subsequent death of the tree. A *Phytophthora* species, tentatively identified as *P. cambivora*, was isolated from bark lesions as well as from soil surrounding dying trees. *Phytophthora cambivora* is a common pathogen on hardwood trees in Europe (Santini et al. 2001; Brasier et al. 2004) and was reported killing maple trees in eastern United States (Pirone, 1940; Crandall et al. 1945), but almost nothing is known about *P. cambivora* on golden chinquapin or in western forests in general.

This study is the first to examine in detail the host-pathogen system of golden chinquapin and this *Phytophthora* species. The primary objective of this study was to confirm the identity of the suspected pathogen, and to experimentally demonstrate its pathogenicity to golden chinquapin. Confirmation of the hypothesis that golden chinquapin is susceptible to *P. cambivora* provides the first step to understanding the epidemiology of this new canker disease for forest managers.

In order to test the hypothesis that golden chinquapin is susceptible to *P. cambivora* experiments were performed to:

1) Confirm the identification of isolates recovered from forest soil around healthy and diseased golden chinquapin trees and from infected bole tissue using morphological comparisons as well as molecular approaches.

2) Complete Koch's postulates by inoculating live trees in the field as well as inoculating seedlings under greenhouse conditions.

2.2 Materials and Methods

2.2a. *Isolation methods*

The three isolates used in this research were collected from 2001-2002 in forested areas within the range of golden chinquapin in Oregon. Two isolates were recovered from forest soil collected at the base of recently killed or healthy chinquapin trees. Approximately 100 g of soil were deposited into a metal baking pan and flooded with 1 L distilled water. Each soil was then baited with 5, one-inch-long pieces of Port-Orford-cedar (*Chamaecyparis lawsoniana*) foliage, and with one partially submerged pear. Soils were baited for 5-6 days at room temperature (~25°C) in the dark. Necrotic foliage baits and necrotic spots on pears were plated onto the *Phytophthora* selective medium CARP^{+} (17 g / L cornmeal agar, with 200μg ml^{-1} ampicilin, 10 μg ml^{-1} rifampicin and 10 μg ml^{-1} pimaricin amended with 50 μg ml^{-1} hymexezol). Growing colonies with morphology resembling *Phytophthora* species were subcultured onto carrot agar (CA) (Kaosiri et al. 1978; Vettraino et al. 2000; Brasier and Kirk, 2004) and maintained at room temperature for further identification and pathogenicity testing.

A third isolate was recovered from an active advancing lesion in the inner bark of a fading golden chinquapin tree. Small pieces of necrotic tissue were plated on CARP+ and observed for developing colonies. Colonies looking like *Phytophthora* were then transferred to carrot agar for further identification. Table 2.1 summarizes the origin of the isolates.

Table 2.1. Isolates of *Phytophthora* spp. collected and used in this research

ISOLATE	*SOURCE OF ISOLATE*	*GEOGRAPHIC LOCATION*	*YEAR COLLECTED*	*COLLECTED BY*
4048	Bark	Butte Falls, OR. USA	July, 2001	E.M. Hansen, D. Goheen, A. Saavedra
4074	Soil	Samtiam Hy., OR. USA	August, 2001	E.M. Hansen
0917-2	Soil	Butte Falls, OR. USA	September, 2002	A. Saavedra

2.2b. *Isolate identification*

Isolates were identified by comparing growing colonies and their reproductive structures against known *Phytophthora* isolates and descriptions of *Phytophthora* species reported in the literature (Newhook et al. 1978; Stamps et al. 1990; Erwin and Ribeiro, 1996). To promote sporangial formation, mycelial plugs, approximately 5 mm diameter, were taken from actively growing colonies of each isolate growing on CA. Plugs were placed into sterile 9 cm petri dishes and flooded with ten ml clarified V8 broth (200 ml of clarified V8 juice diluted into 800 ml of deionized water, Zentmyer et al. 1976) and incubated for two days at 18°C (Ito and Kudo, 1994). Each plate was rinsed 3-5 times using deionized water and then flooded with 10 ml of soil extract water (1g soil / 100 ml deionized water) and incubated for approximately 60 hours.

To determine mating type, the three chinquapin isolates recovered in this research were paired with known tester isolates of *Phytophthora cambivora*, (P31-A2 and P32-A1, courtesy of Sabine Werres, Braunschweig, Germany, and PC-98-1116-A1 from Portland, OR, courtesy of the OSU Plant Disease Clinic), and *Phytophthora cinnamomi*, (PC-98-224- A2 from Gaston, OR, courtesy of the OSU Plant Disease Clinic, and P33-A1 from

Sabine Werres in Germany). All isolates were grown in CA for five days in the dark at room temperature before pairing. Mycelial plugs from the active growing margin of the various isolates were paired in all combinations on CA in 9 cm petri dishes. Isolates were placed 2 cm apart and incubated at room temperature in the dark for 7-8 days, with two replicates of each pairing (Kellam and Zentmyer, 1986).

For further confirmation of the identities of the three chinquapin isolates, DNA from the internal transcribed spacer (ITS) region of the nuclear ribosomal gene was extracted, amplified, and sequenced. A 5 mm dia. plug was taken from colonies growing on CA and DNA was extracted as described in Winton and Hansen (2001).

PCR was performed in 50µl reactions (1x buffer, 200nM dNTP, 0.4 µM ITS4 and ITS5 primers (White et al. 1990), 0.05 U/ µl RedTaq DNA polymerase (Sigma St. Louis, MO) and 2 µl template DNA). The reaction conditions were: 60 s at 94°C, 35 cycles of 60 s at 94°C, 60 s at 55°C, 60 s at 72°C and a final incubation for 7 min at 72°C. The PCR products were prepared for sequencing by adding 0.5µl EXOSAP-IT (USB Cleveland, OH) and incubated overnight at room temperature (~22°C) followed by 15 min at 80°C. Direct sequencing of PCR products (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, CA) was performed with primers ITS4 and ITS5 and run on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Contigs were assembled and edited with the Staden (1996) software package. Edited sequences were compared to sequences of the known species available at GenBank with the BLAST search utility (Altschul et al. 1997)

For determining optimal growth temperatures, isolates were grown on CA in 9 cm petri dishes for two days at room temperature in the dark. After this incubation period, the

plates were moved to incubators at temperatures ranging from 5°C to 35°C for 4 days. Growth rate was determined by measuring colony radius along two lines intersecting at right angles at the centre of the inoculum (Jung et al. 1999; Werres et al. 2001; Jung et al. 2002; De Cock and Lévesque, 2004). Colony margins were marked every two days and the average daily radial growth calculated. Three replicate plates per isolate/temperature were used for this test.

2.2c. *Pathogenicity tests*

Tree inoculation trial

A field inoculation trial was conducted on the Rogue River National Forest, Butte Falls Ranger District, in an area where *P. cambivora* has been recovered from soil and from dying golden chinquapin trees (Table 2.1). This area, located on Oregon State Highway 140 in southwestern Oregon between milepost 28 and milepost 30 has a larger number of dead or dying golden chinquapin trees than any other area surveyed within the State of Oregon (D. Goheen and A. Saavedra personal observations).

Ten visually healthy trees were selected for this study (approximately of 20 cm average diameter at breast height). Trees were inoculated the last week of June 2003. Mycelial plugs from 7 day-old colonies of isolates 0917-2, 4048 and 4074, and agar plugs from uninoculated CA plates were used as inoculum. Holes (5 mm dia.) were punched through the bark to the cambium with a cork borer, the agar plug was inserted and the bark replaced. A moist piece of cheese cloth was placed over the inoculation site and covered with aluminum foil secured in place with tape (Brasier and Kirk, 2001; Hansen et al. 2005). The isolates and the control were randomly placed around the bole at cardinal

directions and at either 15 or 45 cm from the soil surface. Five weeks after inoculation tree outer bark was removed using a drawknife to expose the phloem. Lesions in the inner bark were measured horizontally and vertically from the inoculation point and the lesion area was then calculated.

Seedling inoculation trial

Due to lack of sources of seeds or seedlings of golden chinquapin for this study, seed was collected in the wild. During the months of October and November 2003, hundreds of fruits from several trees and shrubs of golden chinquapin in Oregon and northern California, were collected (Table 2.2)

Table 2.2 List of trees and shrubs of golden chinquapin and locations were fruits were collected

Tree ID	*Location*	*Latitude*	*Longitude*	*Tree/shrub*	*Date collected*
LA-1	Linn Co., OR	N 44.25376	W 122.36595	Tree	Oct. 1, 2003
LB-1	Siskiyou Co., CA	N 41.95817	W 123.12238	Tree	Oct. 8, 2003
LB-2	Siskiyou Co., CA	N 41.94930	W 123.11497	Tree	Oct. 8, 2003
LC-2	Jackson, Co., OR	N 42.31378	W 122.29383	Shrub	Oct. 9, 2003
LD-1	Jackson, Co., OR	N 42.38452	W 122.36751	Tree	Oct. 9, 2003
LD-2	Jackson, Co., OR	N 42.38784	W 122.35649	Tree	Oct. 9, 2003
LD-1 (1028)	Jackson, Co., OR	N 42.38452	W 122.36751	Tree	Oct. 28, 2003

Collected fruits were dried and nuts extracted at USDA-Forest Service Dorena Genetic Resource Center. Nuts that were released from the burrs after the drying process were checked for damage, mainly caused by insects. Clean seeds were planted in 5 cm dia. x

17 cm depth Deepots™ (Hummert™ International Earth City, MO) in the greenhouse at Dorena.

Only 60 seeds germinated out of several hundred planted. After 8 months, the surviving seedlings were transported to an Oregon State University greenhouse. At this facility they were maintained and prepared for the inoculation trials.

Two months before the trial, seedlings were observed to be infected with powdery mildew. They were sprayed twice with a 1:1 (v/v) solution of Quintec (Dow AgroSciences), a protective fungicide used for the control of powdery mildew on grapes and hops (Pscheidt and Ocamb, 2005), and Kaligreen™ (Toagosei, Co., LTD, Japan), a potassium bicarbonate salt that in trials has reduced the infection by powdery mildew in flowering dogwood (Mmbaga and Sheng, 2002; Mmbaga and Suavé, 2004).

Seedlings were inoculated with a zoospore suspension from isolates 4048 and 4074. To induce sporangia, mycelial plugs from 7-day-old colonies on CA were placed into sterile 9 centimeter petri dishes and flooded with ten ml clarified V8 broth and incubated for two days at 18°C (Ito and Kudo, 1994). Each plate was rinsed 3-5 times using deionized water and then flooded with 10 ml of soil extract water (1g soil / 100 ml deionized water) and incubated for another two and one half days.

To induce zoospore release, the soil extract was replaced with cold deionized water (water was chilled in a freezer for ~ 1 hour) and the plates were incubated for 2 hours at 4°C then returned to room temperature. The concentration of released zoospores was determined using a (Spencer Bright-line) hemacytometer (AO Instrument Co., Scientific Instrument Division. Buffalo, NY. USA) with the help of a light microscope. The zoospore concentration was calculated to be $1x10^4$ per ml.

On August 1st, 2005, 40 one-year-old chinquapin seedlings growing in pots were selected to be inoculated with the two isolates grown for this trial. A hole in the soil of each pot was opened using a glass rod. Ten ml of zoospore suspension from each isolate were added to each pot. Ten additional seedlings that served as controls were treated with 20 ml of one-percent soil extract. All seedlings were immediately flooded with tap water. After 24 hours the flooding water was drained and the seedlings were watered twice every day for 19 days and then the pots were flooded again for another 24 hours. After that plants were watered twice a day again. Seedling mortality was recorded daily for the duration of the study. During and after the experiment dead seedlings were examined for rotting roots and inner bark stain. Diseased tissues were plated in $CARP^{+}$, incubated in the dark at room temperature and observed under the light microscope.

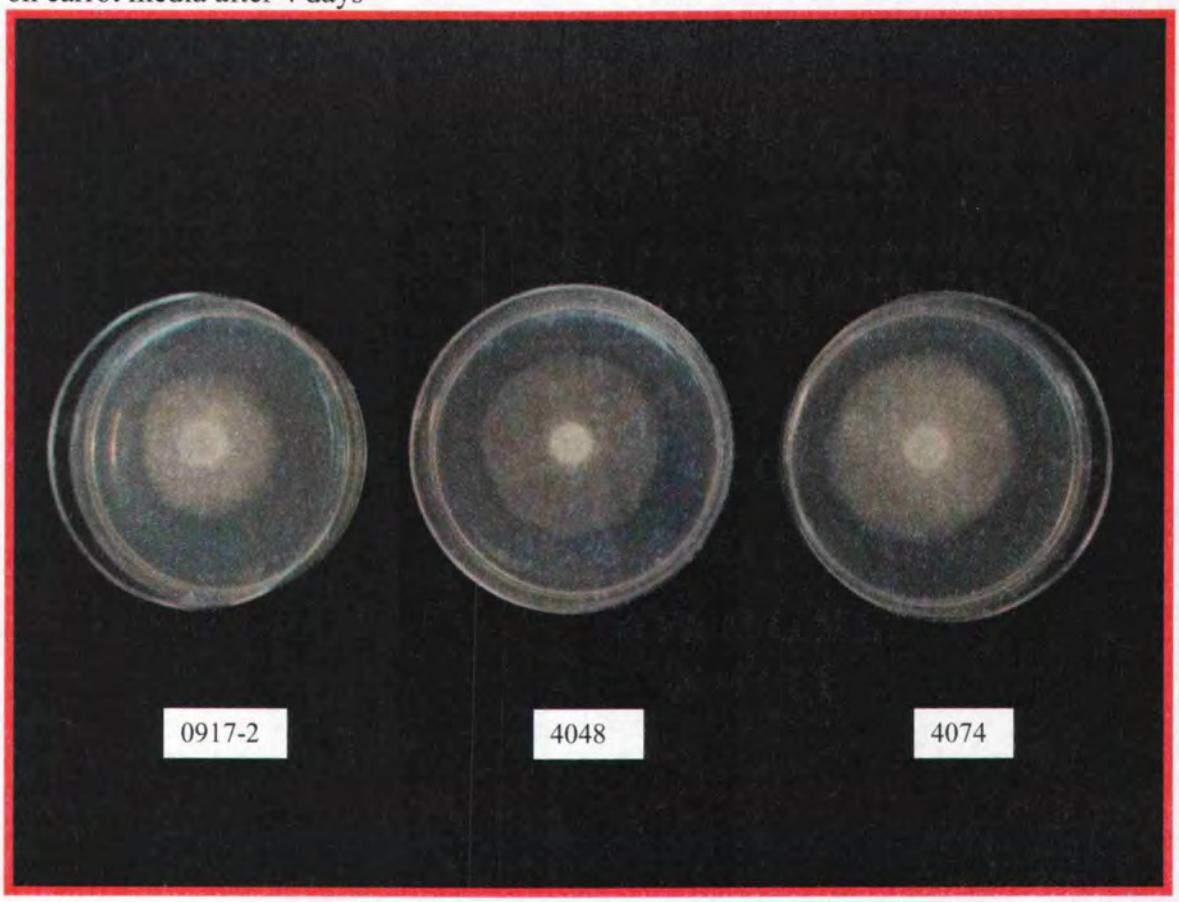
2.3 Results

2.3a. *Isolate identification*

Colony description

Colony morphology of the chinquapin isolates in this study closely resembled the tester isolates and the published descriptions of *P. cambivora* (Waterhouse, 1956; Waterhouse, 1963; Erwin and Ribeiro, 1996). Colonies growing in CA were cottony, with moderate to profuse aerial mycelium and no particular pattern in the agar (Fig 2.1). The mycelia were coralloid (Fig 2.2a) with distinct hyphal swellings.

Fig 2.1. Colony morphology of isolates of *Phytophthora cambivora* at room temperature on carrot media after 4 days



Mating type testing

Mating type pairing revealed both A1 and A2 mating types among the chinquapin isolates from Oregon forests. The two isolates recovered from the Butte Falls area (Table 2.1) formed reproductive structures when paired against known A1 isolates of *Phytophthora cambivora* and *P. cinnamomi* after a little more than a week growing in CA . The resulting oogonia with "warts" and the two celled amphigynous antheridia morphologically resembled those of *P. cambivora* (Fig 2.2a, b, c). It was concluded that isolates 0917-2 and 4048 were mating type A2. Isolate 4074, which was recovered from

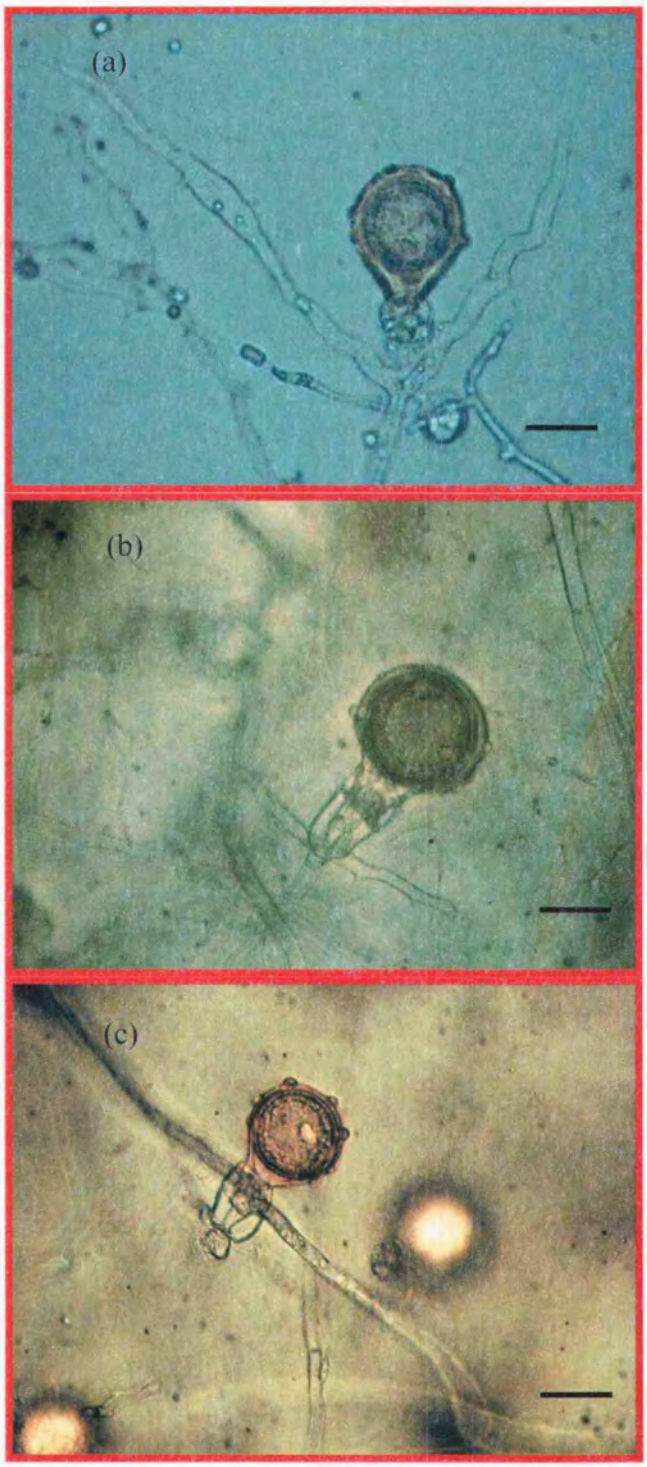
soil from a stand with healthy golden chinquapin trees approximately 150 miles north of Butte Falls, did not form oogonia when paired with any of the tester isolates. It did form sexual structures when paired with the other two chinquapin isolates, 4048 and 0917-2, however. As a result, we concluded that isolate 4074 was mating type A1.

Oogonial diameters were measured for 50 oogonia from each pairing test. Average oogonial diameter of isolate 0917-2 was 40.7 μm; oogonial diameters of isolate 4048 averaged 43.3 μm and isolate 4074 averaged 43.8 μm. All of these measurements are close to those described by Stamps et al. (1990) and Erwin and Ribeiro (1996) for *Phytophthora cambivora*. Table 2.3 summarizes the characters of the reproductive organs obtained from this study.

Table 2.3 Comparison of the characters of the isolates from this study and those reported in the literature

		STAMPS ET AL. 1990	*ERWIN AND RIBEIRO 1996*	*CURRENT STUDY*
Oogonia	diameter (μm)	40-50	43-63	41-44
	morphology	ornamented	ornamented	ornamented
Antheridia	position	amphygynous	amphygynous	amphygynous
	No. cells	2	2	2
Sporangia	length (μm)	> 75	40-75	65
	morphology	ovate, ellipsoid	ovate, ellipsoid	ovate, ellipsoid
	papillate	non	non	non
Colonies	hyphal swellings	present	present	present
	growth temperature(°C)	23-27	22-24	20-25

Fig 2.2 Amphigynous antheridia and oogonia of isolates of *Phytophthora cambivora* (a=isolate 0917-2; b = isolate 4048; c=isolate 4074. Bar = 30 microns)



Sporangia description

After two and one half days incubation at 18°C in soil extract water in the dark, isolates 4074 and 4048 formed sporangia in abundance. Isolate 0917-2 also formed sporangia but only after 4-5 days incubation and sporangia were not as numerous as the other two isolates. Sporangia for all three isolates were non-papillate, ovoid in shape, non-caduceus and with an average length of 65 μm.

d. Optimal growing temperature

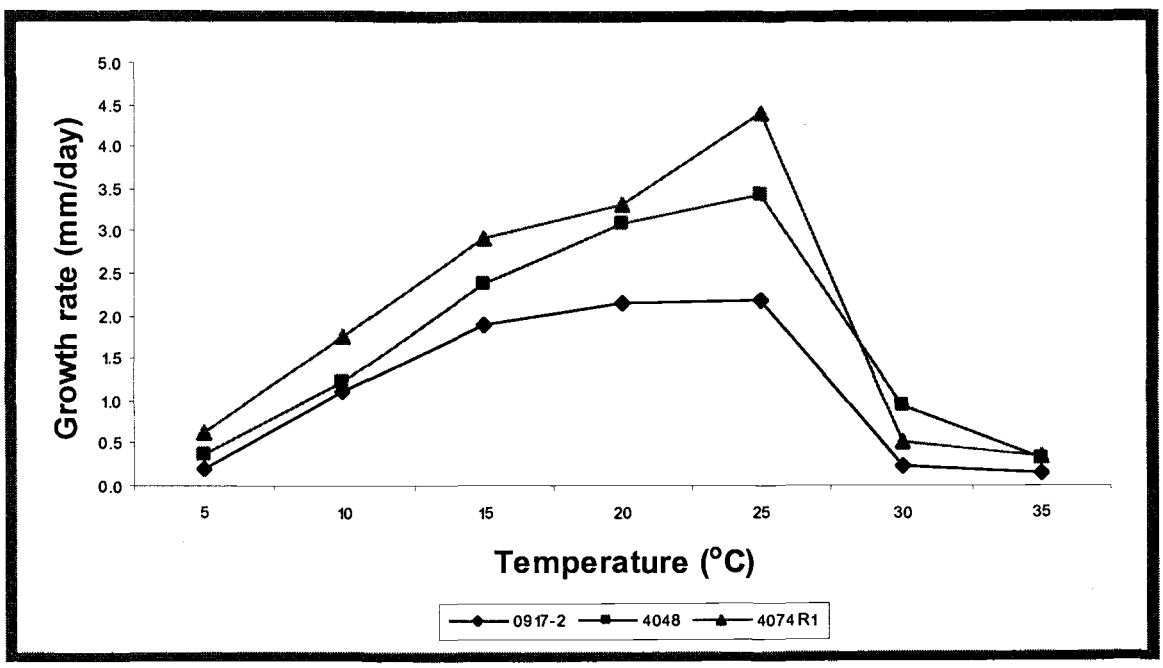
Optimal growing temperature for the chinquapin isolates was 25°C (Table 2.4 and Fig 2.3). At this temperature, isolates grew from 2.2 to 4.4 mm/day on CA. All isolates grew at 5°C and 35°C, but much more slowly. These growth characteristics are comparable to published values for *P. cambivora* (Table 2.3).

Table 2.4. Comparison of growth rate among isolates of *P. cambivora* in this study

Name of Isolate	Temperature (Celsius)	mm [1]	mm [2]
0917-2	5	0.4	0.2
	10	2.2	1.1
	15	3.8	1.9
	20	4.3	2.1
	25	4.4	2.2
	30	0.5	0.2
	35	0.3	0.1
4048	5	0.8	0.4
	10	2.4	1.2
	15	4.8	2.4
	20	6.2	3.1
	25	6.9	3.4
	30	1.9	0.9
	35	0.6	0.3
4074	5	1.3	0.6
	10	3.5	1.8
	15	5.8	2.9
	20	6.7	3.3
	25	8.8	4.4
	30	1.0	0.5
	35	0.7	0.3

[1] Mean of 2 days growth rate along 4 radii from 3 plates per isolate
[2] Mean daily growth rate along 4 radii from 3 plates per isolate

Figure 2.3. Mean daily growth rate (mm) from three plates per 3 isolates of *P. cambivora* tested in this study



d. ITS rDNA sequencing

The rDNA- ITS sequence of the chinquapin isolates matched from 99% to 100% with GenBank sequences of *Phytophthora cambivora*. Isolate 0917-2 and 4048 were 100% homologous (619 of 619 bps and 627 of 627 bps respectively) with *Phytophthora cambivora* isolate AG45 clone 2 (GenBank accession number AY787029). Isolate 4074 was 99% homologous with AG45 clone 2 (783 of 784 bps). For isolates 0917-2 and 4074, nine of ten best sequence matches from GenBank were for *P. cambivora* accessions. The only exception was a sequence for *Phytophthora alni* subsp. *multiformis* (GenBank accession number AY689136.1). For isolate 4048, besides *P. alni* subsp *multiformis*, *Phytophthora fragariae* was among the top 10 returns. *P. fragariae* and *P. cambivora* are presumably parents of hybrid *P. alni*. Although the latter also possesses ornamented oogonia and it is closely related to *P. cambivora* (Brasier et al. 2004), *P. alni* is a homothallic species whereas *P. cambivora* is a heterothallic species like the chinquapin-related isolates. *Phytophthora fragariae*, is closely related to *P. cambivora* but, it doesn't have an ornamented oogonium and it is a homothallic species (Stamps et al. 1990; Cooke et al. 2000).

2.3b. *Pathogenicity testing*

Tree inoculation trial

Five weeks after inoculations, the bark of inoculated golden chinquapin trees was removed using a draw knife, revealing areas of necrotic phloem around the inoculation points (Fig 2.4). Lesions were dark brown and diamond shaped. A limited area of necrotic phloem was observed around control wounds. Control lesion area averaged 1.1

cm^2. The mean lesion area for the inoculated isolates was 171.4 cm^2. *Phytophthora* was reisolated on CARP+ from lesions on 8 out of 10 inoculated trees. Recovered isolates were morphologically similar to colonies of the same isolates growing in the laboratory.

Measurements of the lesions on the inner bark were collected and lesion area calculated (Fig 2.5; Table 2.5). Analysis of the effect of the location of the inoculations in trees (15 cm from ground or 45 cm from ground) and the aspect of inoculations (N, S, E, or W) showed no significance (p-values 0.27 and 0.77 respectively), so the data were pooled for further analysis. The difference in lesion area between inoculated wounds and control wounds was significant (p-value = 0.000014) (Fig 2.6; Table 2.6). However, there was no significant difference in lesion area caused by the isolates used in this study (Fig 2.7; Table 2.7)

Fig 2.4. Bark lesions on bole of golden chinquapin trees: (a) bark lesion caused by *P. cambivora* isolate inoculated in bark; (b) control, plain carrot agar in same tree

Table 2.5. Bole lesion area (cm^2) by tree per isolate of *P. cambivora* inoculated in the bark of ten golden chinquapin trees

Tree identification	*Isolate identification*	*Location in the bole*	*Aspect*	*Length (cm)*	*Width (cm)*	*Lesion[1] area (cm^2)*
M1	4074	Lower	South	35.1	10.3	180.8
	0917-2	Upper	West	41.8	10.3	215.3
	4048	Upper	East	39.1	12.9	252.2
	ctrl	Lower	North	0.4	0.3	0.1
M2	4074	Upper	West	20	2.2	22.0
	0917-2	Lower	North	22.5	5.5	61.9
	4048	Upper	East	18.7	16.3	152.4
	ctrl	Lower	South	0	0	0.0
M3	4074	Lower	East	24.8	21.5	266.6
	0917-2	Upper	North	27	19.7	266.0
	4048	Upper	South	27.1	25.5	345.5
	ctrl	Lower	West	0	0	0.0
M4	4074	Upper	South	41.4	17.7	366.4
	0917-2	Upper	North	38.9	16.1	313.1
	4048	Lower	West	28.3	16.3	230.6
	ctrl	Lower	East	0	0	0.0
M5	4074	Lower	East	26.5	7.5	99.4
	0917-2	Upper	South	28	11	154.0
	4048	Upper	North	30	9.6	144.0
	ctrl	Lower	West	5.2	4.1	10.7
T1	4074	Upper	South	37.4	10.9	203.8
	0917-2	Upper	North	36.2	9.7	175.6
	4048	Lower	East	20.1	5.7	57.3
	ctrl	Lower	West	1.1	0.2	0.1
T2	4074	Upper	East	25.5	9.2	117.3
	0917-2	Lower	South	32.8	10.5	172.2
	4048	Lower	North	29.7	10.4	154.4
	ctrl	Upper	West	0.4	0.2	0.0
T3	4074	Upper	South	38.3	7.3	139.8
	0917-2	Upper	North	20.7	8.7	90.0
	4048	Lower	West	33.7	6.4	107.8
	ctrl	Lower	East	0.9	0.4	0.2
T4	4074	Upper	West	35.1	14.4	252.7
	0917-2	Lower	South	24.8	8.2	101.7
	4048	Upper	East	33.3	9.9	164.8
	ctrl	Lower	North	0.3	0.2	0.0
T5	4074	Lower	West	26.1	14.8	193.1
	0917-2	Upper	South	19.7	11.1	109.3
	4048	Upper	North	31.3	2.1	32.9
	ctrl	Lower	East	0	0	0.0

[1] Lesion area is the product of length x width divided by 2

Figure 2.5. Basal lesion area (cm^2) by tree per isolate of *P. cambivora* inoculated in the bark of ten golden chinquapin trees

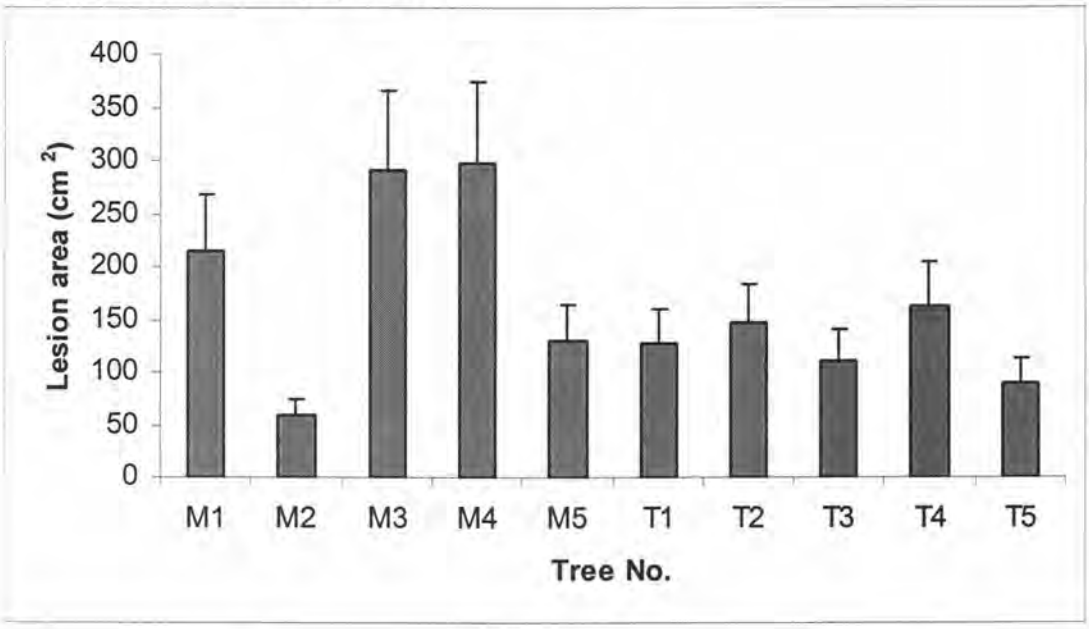


Table 2.6. Analysis of variance for isolate effect (including the control as an isolate) on mean lesion area in inoculated boles of *Chrysolepis chrysophylla*

Source	df	Ms	*F*	*P*
Isolates	3	73344.27	11.94495	0.000014
Residuals	36	6140.19		
Total	39	79484.46		

Fig 2.6. Mean lesion area on ten boles of golden chinquapin inoculated with three isolates of *Phytophthora cambivora*

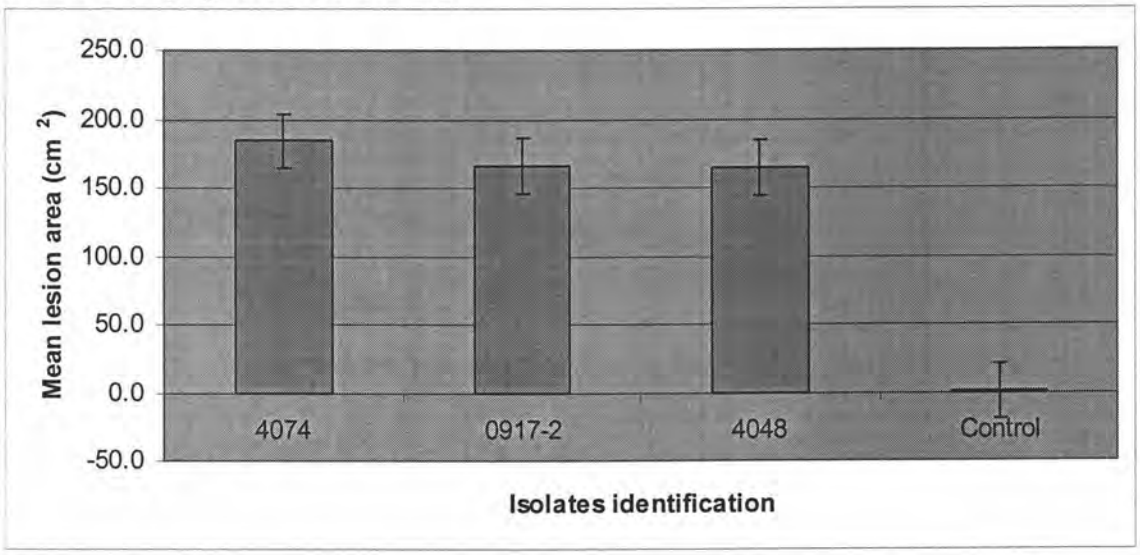
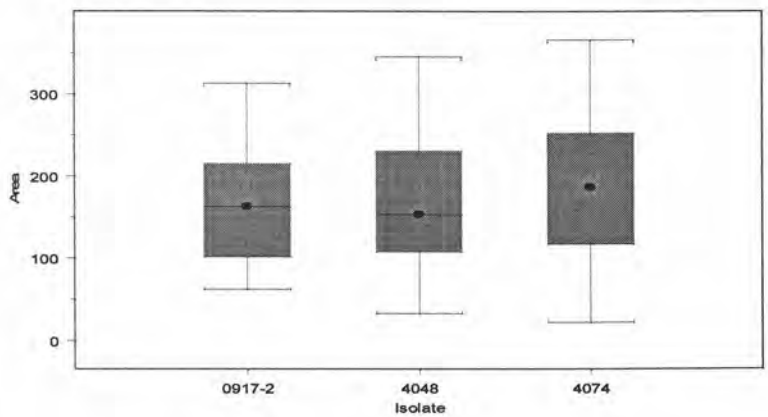


Table 2.7. Analysis of variance for isolate effect on mean lesion area on inoculated boles of *Chrysolepis chrysophylla* with isolates of *P. cambivora* (control not included)

Source	df	Ms	*F*	*P*
Isolates	2	1227.9	0.15	0.86
Residuals	27	8183.2		
Total	29	9411.1		

Figure 2.7. Mean lesion area (cm^2) of 3 isolates per ten trees



Analysis of variance showed that there was a significant difference in lesion area between trees (Fig 2.5; Table 2.8) with a p-value of 0.000933.

Table 2.8. Analysis of variance for tree effect on mean lesion area in boles of *Chrysolepis chrysophylla* inoculated with isolates of *P. cambivora* (control not included)

Source	df	Ms	*F*	*P*
Tree No.	9	17488.1	5.30	0.000933
Residuals	20	3300.4		
Total	29	20788.5		

Seedling inoculation

100% of seedlings that were soil inoculated with isolates 4048 and 4074 were dead after 38 days under greenhouse conditions (Fig 2.8; Table 2.9); there was no mortality among control seedlings that were treated with soil extract water only. First symptoms were visible after 11 days. Symptoms included yellowing of leaves that later turned brown and either dropped or remained on stems but dried. The roots were completely brown and rotted in comparison to the roots of the control seedlings that showed no necrotic symptoms (Fig 2.9). Observations of the inner bark of roots showed necrosis caused by the inoculated isolates. Examinations of the inner bark of stems also showed lesions red to brown in color and girdling the stems. The lesions were determined to be coming from the main roots of inoculated seedlings.

Fig 2.8. One-year-old golden chinquapin seedlings 38 days after inoculation with isolates of *P. cambivora* under greenhouse conditions: a) control seedling; b) inoculated seedling

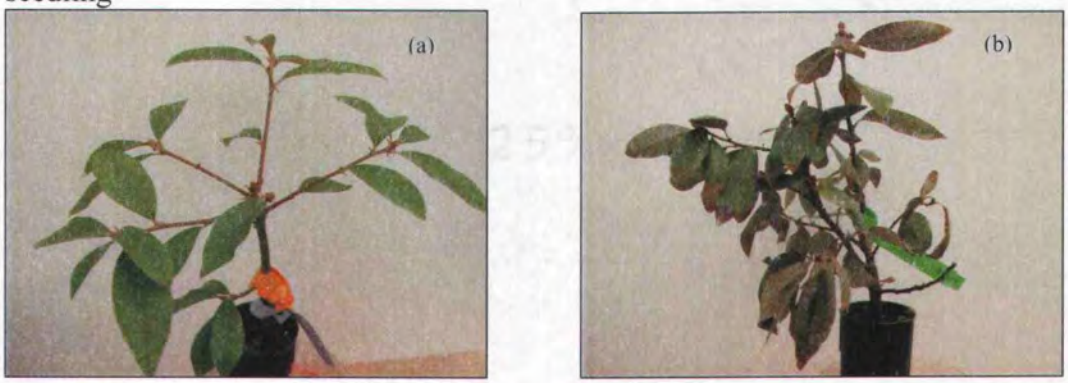


Fig 2.9. Root conditions of one-year-old golden chinquapin seedlings 38 days after inoculation with isolates of *P. cambivora* under greenhouse conditions: a) control seedling; b) inoculated seedling

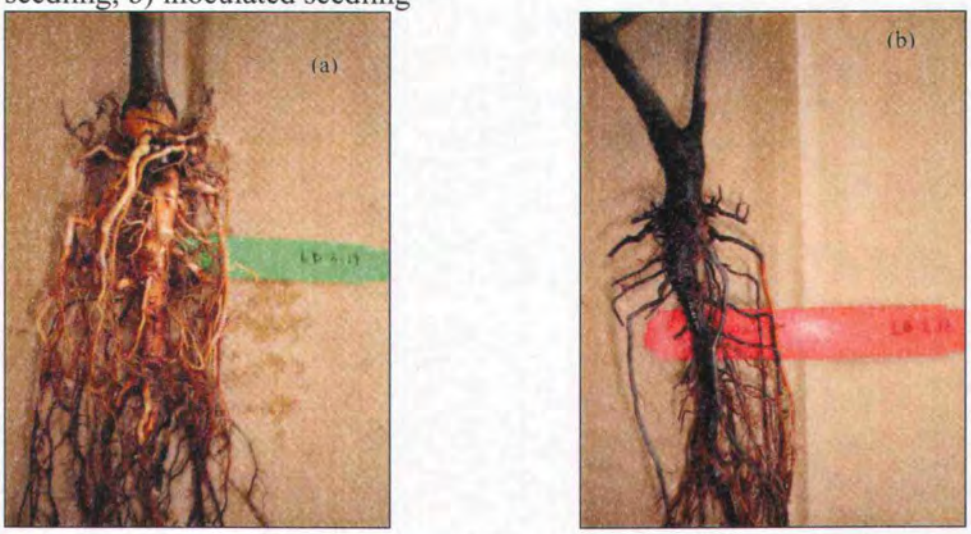


Table 2.9. Mortality of golden chinquapin seedlings inoculated with isolates of *P. cambivora*

	Number of seedlings	Dead	Alive
Inoculated seedlings	40	40	0
Control seedlings	10	0	10

2.4 Discussion

The results of this study confirm that the *Phytophthora* species isolated from Oregon forest soil and from dying golden chinquapin trees is *Phytophthora cambivora* (Petri) Buisman. Morphological characteristics of these isolates agreed with those of *P. cambivora*. Colonies were moderate to fast growing with no specific patterns and with aerial mycelium which gave them a cottony appearance. Mycelium presented numerous hyphal swellings and no chlamydospores were observed. Oogonia were distinctively ornamented with a diameter ranging from 40.7 μm to 43.8 μm and the two celled antheridia were amphigynous. Sporangia, which were produced in large numbers, were non papillate, ellipsoid to ovoid, non deciduous, with an average length of 65μm.

The chinquapin isolates showed some variation in growth rate at different temperatures. Isolate 4074, which was recovered from soil in the northern range of golden chinquapin, had a faster growth rate than isolate 0917-2 from Southern Oregon which was also recovered from soil. All three isolates showed little growth at 5°C and above 30°C. The optimal growing temperature ranged between 20°C to 25°C as has been reported for this species (Erwin and Ribeiro, 1996). Both mating types were morphologically similar and their growth rate was affected in the same manner depending upon the temperature. It can't be determined from the isolates used in this study that mating type A1 of *Phytophthora cambivora* is a faster grower than A2 as has been reported for other species of *Phytophthora* (Brasier, 2003). In addition, the ITS rDNA sequencing results confirmed that the isolates recovered from soil, whether surrounding healthy or declining golden chinquapin, as well as the isolate recovered from an active lesion margin belong

to *P. cambivora* as they matched from 99% to 100% bp with GenBank sequences of *Phytophthora cambivora* (AG45 clone 2).

There have been other reports of *Phytophthora cambivora* in the Pacific Northwest. Researchers in Oregon and Washington have associated *P. cambivora* with root rot and stem canker in Noble fir in Christmas tree plantations (Hamm and Hansen, 1987; Chastagner et al. 1995). In addition, *Phytophthora cambivora* has been recovered from forest soil around Douglas-fir trees with basal cankers (Hansen pers. comm.). Isolates used in the present study were from forest settings associated with this new canker disease on golden chinquapin as well as from forest soil around apparently healthy chinquapins in an area where unusual mortality has not been noted.

Both mating types of *P. cambivora* have been reported from the Pacific Northwest. Mating type A1 of *P. cambivora* was frequently recovered from dead roots and stem cankers in noble fir Christmas tree plantations (Hansen pers. comm.). In addition, an isolate of mating type A2 from noble fir in Oregon (ATTC 46719) was cited by Oudemans and Coffey (1991) in their isozyme comparison study, and in the present study, both mating types of *P. cambivora* were recovered from Oregon forest soil in geographically distant locations. Further surveys across the range of golden chinquapin will be needed to verify the distribution of *P. cambivora* mating types.

Results of tree and seedling inoculations support the original hypothesis that golden chinquapin is susceptible to *Phytophthora cambivora*. The method used for inoculating seedlings, adding zoospore suspension directly into the soil, gave results comparable to other pathogenicity studies with *P. cambivora* (Jung et al. 2003; Jung et al. 2005). In these studies, *Phytophthora cambivora* was incorporated into the soil and caused root rot

and mortality (85-100% and 70-90 to 100% respectively) on seedlings of European beech (*Fagus sylvatica* L.). The current study showed that seedlings of golden chinquapin were also highly susceptible (100% mortality) to *P. cambivora* under greenhouse conditions. Examinations of roots showed that they were highly rotted and their biomass significantly reduced (Fig 2.9) compared to control roots. In addition, extensive necrotic lesions were also found in the inner bark of every killed seedling. Because isolates were mixed for the seedling inoculations, differences in aggressiveness of the isolates of *P. cambivora* could not be addressed in this study. Recovery of *P. cambivora* from inoculated seedlings successfully completed an objective of this study by satisfying Koch's postulates. However, it is also important to note that the seedlings used in this study represented a very limited portion of the possible variation of responses that a larger population of golden chinquapin seedlings might have provided. Due to the lack of availability of seedlings from commercial nurseries and the low rate of seed germination, only 50 seedlings from four specific areas that were readily accessible by roads were used in this study. A more detailed study representing a more diverse population from the entire range of golden chinquapin is necessary to answer more questions regarding the level of susceptibility of golden chinquapin to *P. cambivora.*

A short study during the summer of 2002 (Saavedra and Goheen; no data included in this work) collected data regarding the behavior of this disease in the area in which *P. cambivora* is causing the most damage. This study suggested a relationship between road proximity and incidence of the disease. Several surveys conducted during the following two summers within the range of golden chinquapin also suggested that the disease might be related to road proximity.

Turchetti (1988) reported that *P. cambivora* penetrated chestnuts through the fine roots. The results of drenching zoospores directly into the soil suggest that penetration of this pathogen into seedlings of golden chinquapin is also through fine roots. Observations of the lesions on stems in the forest supported this possibility, since the infection on the stem was always traceable to infected main roots. The mechanism of penetration of encysted zoospores into the fine roots of golden chinquapin cannot be explained in this current work but a study described by Casares et al. (1994) reported that hyphae of *P. cambivora* grew intercellularly in callus cells of European chestnut (*Castanea sativa* Miller) by the action of degrading enzymes produced at the tip of advancing hyphae and also intracellular infection of callous cells via haustorial formation. This study could provide insight to explain modes of infection of *P. cambivora* into the roots of golden chinquapin seedlings.

Field inoculation trial results also confirmed the hypothesis that golden chinquapin is susceptible to *P. cambivora*. Lesions were observed in the inner bark of inoculated trees *P. cambivora* was recovered from the margins of active lesions. Although the boles of inoculated trees were almost girdled by the pathogen by the end of the trial period, the trees were still alive. Crown symptoms were observed, however, including leaf chlorosis and some branch flagging. It is believed that the infection caused by this pathogen would have led to the eventual death of inoculated trees. Two years after the end of this trial the inoculated trees were dead (pers. observations) but the mechanical girdling of trees during lesion measurement undoubtedly contributed to the rapid mortality.

There are questions that need to be addressed in further studies for achieving a better understanding of this new disease. Is the apparent correlation between road proximity and tree mortality real? If it is real, is it due to predisposition, or transport of the pathogen along the roads? Another question that remains unanswered is the aggressiveness of this disease. There is little information on how quickly mature trees might be killed after initial infection by *P. cambivora*. Results of this study showed rapid advance of *Phytophthora cambivora* through phloem tissues suggesting that girdling of trees could occur within months of bark infection but does not answer the question of how long it takes for the pathogen to move out of the roots, or how long it takes girdled trees to die.

In summary, *Phytophthora cambivora* is present in Oregon forest soil and both mating types are represented. Both inoculation methods used, bark inoculation of mature living trees or root infection of seedlings growing in soil infested with a zoospore suspension, resulted in the formation of necrotic tissues and in the case of the greenhouse seedling experiment, 100% mortality. In both instances, the pathogen was successfully recovered from the infected tissue satisfying Koch's postulates and demonstrating that *Phytophthora cambivora* is the causal agent of this new root rot and basal canker disease of golden chinquapin.

However, further studies need to be conducted for better understanding of this disease and its ecological impact in Oregon forests.

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Summary and Conclusion

Chapter 3

Phytophthora cambivora has been known as a pathogenic species on trees of economic importance, especially European chestnut, since it was first described by Petri in 1917 (Cited in Tucker, 1933). Later other authors reported this pathogen affecting other tree species of economic and ecological importance (Pirone, 1940; Mircetich et al. 1974; Jeffers and Aldwinckle, 1988; Chastagner et al. 1995; Shafizadeh and Kavanagh, 2005). In all of these reports *P. cambivora* was causing root rot and basal canker to its hosts.

Golden chinquapin, a native tree of the western United States, is not known to be affected by many pests other than a few heart-rotting fungi and a few insect pests (Hepting, 1971; McKee, 1990; Sinclair and Lyon, 2005). This species is very resistant to chestnut blight, caused by *Cryphonectria parasitica*, in contrast to its close relatives, *Castanea* species.

However, in recent years serious pockets of mortality affecting golden chinquapin trees have been observed (D. Goheen pers. comm.). Infected trees showed crown symptoms including leaves turning from green to bright red before being dropped, or branch flagging, with the dead leaves retained . Another symptom was the presence of necrotic tissues in the inner bark of stems and roots.

Initial isolations from active growing cankers and from surrounding soil yielded a *Phytophthora* species morphologically resembling *P. cambivora*. This current study was

undertaken to establish the true identity of the recovered fungus, and to determine its pathogenicity on golden chinquapin by satisfying Koch's postulates.

Several approaches were pursued to identify the isolates. Three chinquapin isolates were characterized and compared to published descriptions. Two of the isolates were recovered from the epidemic area (Butte Falls, OR), one from soil associated with dead trees and the other from necrotic inner bark of an infected golden chinquapin. They were labeled as isolates 0917-2 and 4048 respectively. A third isolate was recovered from soil near healthy-appearing chinquapin trees in the northern range of the tree (Linn Co. OR). This isolate was identified as 4074.

The isolates were grown in carrot agar and their morphological characteristics were compared with those of known isolates of *Phytophthora cambivora*. The chinquapin isolates produced aerial mycelium giving the colonies a cottony appearance, and the hyphae often exhibited coralloid hyphal swellings. These characters were also present on the known isolates of *P. cambivora* and matched those described in the literature (Waterhouse, 1956; Stamps et al. 1990).

To induce sporangia, a technique modified from Ito and Kudo (1994) was used. Numerous sporangia were formed. Sporangia were ellipsoid, non papillate, non caducous, with simple sporangiophores and measured in average 65μm in length.

The isolates' mating types were also resolved by pairing against tester isolates of known mating type. As a result, isolates 0917-2 and 4048 were determined to be of mating type A2 whereas isolate 4074 was determined as mating type A1. The resulting oogonia were ornamented as described for this species (Ho et al. 1977; Stamps et al. 1990) with an average diameter that ranged from 40.7μm to 43.8μm. The antheridia

were two celled and amphigynous, again matching published descriptions for *Phytophthora cambivora*.

Growth rate testing results indicated that the optimal growing temperature for the chinquapin isolates ranged from 20°– 25°C, and that growth was restricted or stopped at extreme temperatures of 5°C and above 30°C. These temperature values agreed with the growing temperatures recorded for this pathogenic species (Erwin and Ribeiro, 1996).

Another approach used for establishing the identity of the chinquapin isolates was sequencing their ITS r-DNA, as used by other researchers for identifying suspected or unknown species (Winton and Hansen, 2001; Hansen et al. 2003; De Cock and Lévesque 2004; Brasier et al. 2005; Greslebin et al. 2005). All three isolates showed high homology with *Phytophthora cambivora* isolate AG45 clone 2 (GenBank accession number AY787029). The molecular analysis in combination with morphological and physiological observations supported the conclusion that all three of the isolates, from dying chinquapin trees and associated soil, and from soil around healthy trees, belonged to *Phytophthora cambivora*.

Pathogenicity tests confirmed that *Phytophthora cambivora* was the casual agent of this new canker disease. All 40 seedlings of golden chinquapin inoculated with zoospore suspensions of *Phytophthora cambivora* died within 38 days. In contrast, all 10 control seedlings that were treated with a soil extract solution remained healthy. *P. cambivora* was recovered from rotting roots and stems of the inoculated seedlings but not from control seedlings, completing Koch's postulates.

Inoculation of living, mature chinquapin trees in the field also demonstrated that *P. cambivora* is the causal agent of canker disease on golden chinquapin. All 10 golden

chinquapin trees inoculated with plugs containing growing mycelia of *P. cambivora* developed cankers within weeks after inoculation. Cankers resembled those seen on dying trees in nature. *P. cambivora* was isolated from lesions on inoculated trees but not from control wounds, satisfying Koch's rules.

In summary, *P. cambivora* has been isolated, identified and confirmed responsible for a canker disease on golden chinquapin in Oregon. This is believed to be the first report of *P. cambivora* causing disease on golden chinquapin.

Future suggestions

For achieving a further understanding of the behavior of this disease, it is suggested:

1) To conduct a more extensive study of the relationship between road proximity and severity of the disease in stands where mortality is evident.

2) To make an extensive survey throughout the natural range of golden chinquapin to determine the impact that this disease may have already caused in nature.

3) To carry out more inoculation trials that include a better representation of golden chinquapin populations for identifying resistant individuals and studying possible mechanisms involved in resistance.

4) To conduct a more detailed soil survey for determining the distribution of *Phytophthora cambivora* in Oregon forests.

5) To evaluate the ecological impact that this disease may cause in Oregon forest communities.

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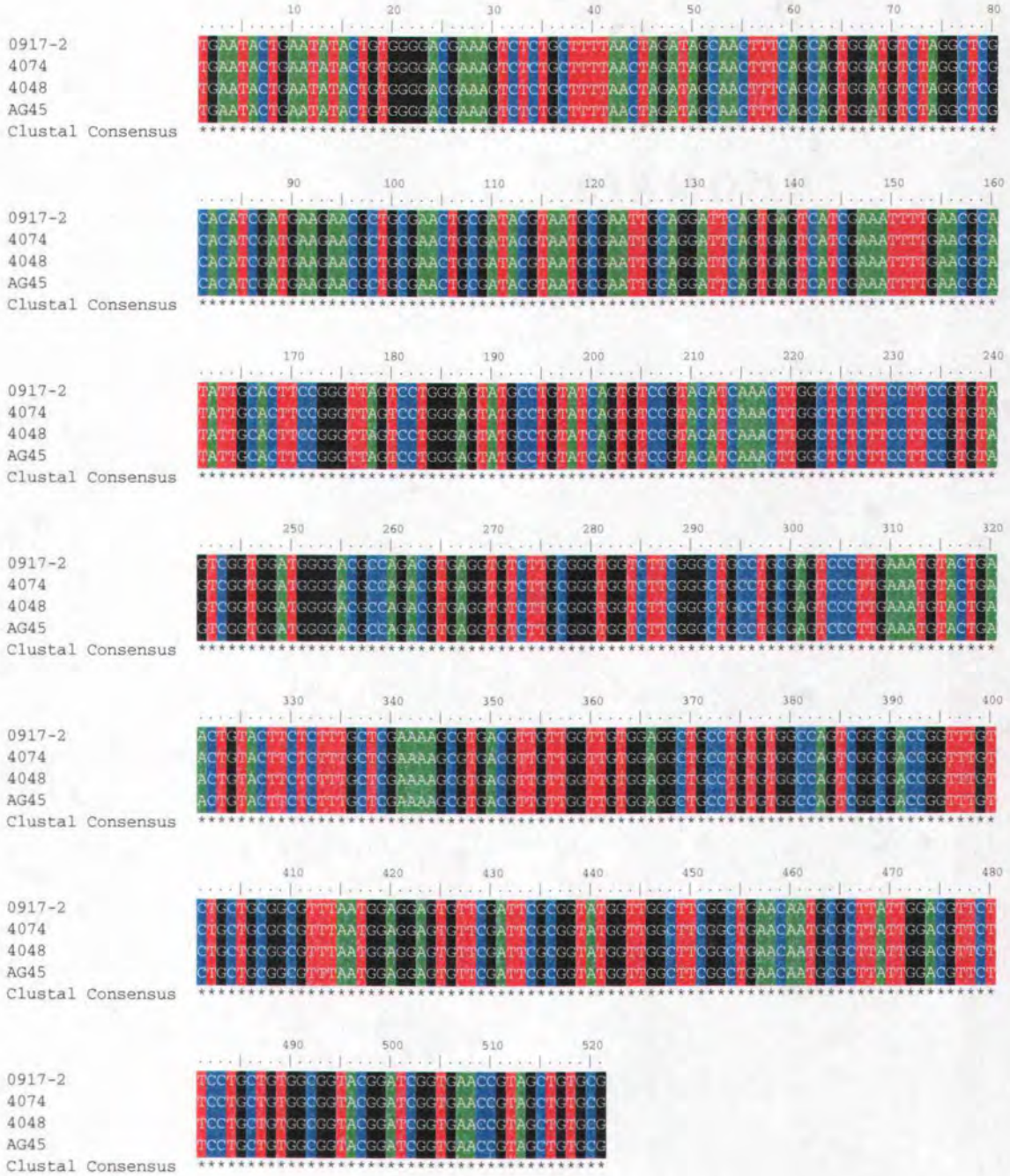
APPENDICES

APPENDIX A

rDNASequence alignments of chinquapin isolates and isolate AG45 clone 2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Clustal Consensus
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Clustal Consensus
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0917-2
4074
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Clustal Consensus
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0917-2
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Clustal Consensus
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0917-2
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Clustal Consensus
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0917-2
4074
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Clustal Consensus
490 500 510 520
0917-2
4074
4048
AG45
Clustal Consensus

APPENDIX B

Pictures of necrotic lesions on ten golden chinquapin trees inoculated with isolates of *Phytophthora cambivora*.

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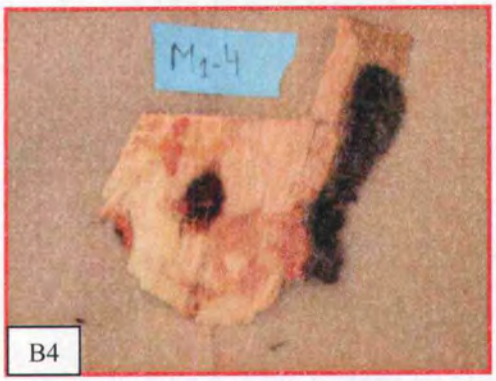

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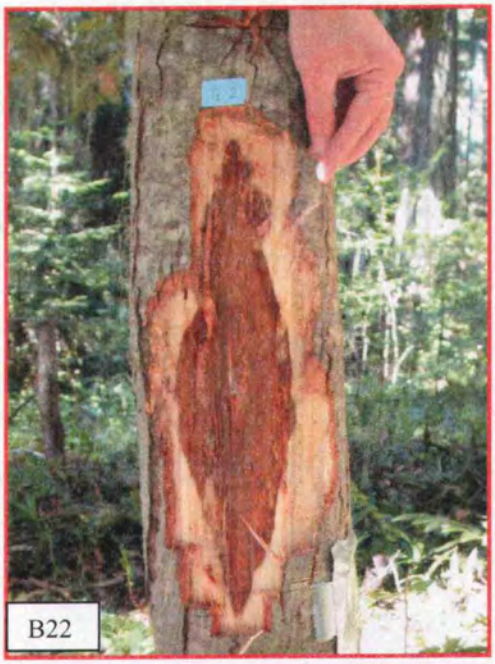
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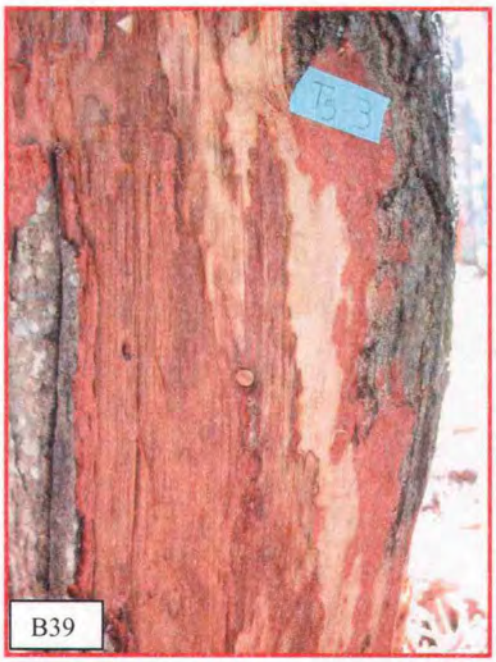

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APPENDIX C

Pictures of golden chinquapin seedlings before and after treatment with isolates of *Phytophthora cambivora* under greenhouse conditions

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APPENDIX C (Continued)

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APPENDIX C (Continued)

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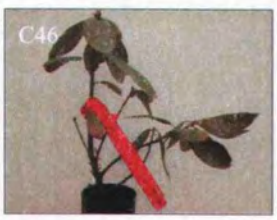
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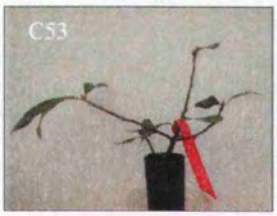
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