Characterization of Oomycete Species Associated With Root and Crown Rot of English Walnut in Chile

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Abstract

English (Persian) walnut (*Juglans regia*) trees affected by root and crown rot were surveyed in five regions of central Chile between 2015 and 2017. In each region, nine orchards, ranging from 1 to 21 years old, were randomly selected and inspected for incidence and severity of tree decline associated with crown and root rot. Soil and symptomatic crown and root tissues were collected and cultured in P₅ARP semiselective medium to isolate potential oomycete pathogens, which were identified through morphology and molecularly using ITS sequences in the rDNA

gene and beta tubulin gene. The most frequently isolated species was *Phytophthora cinnamomi*. Pathogenicity tests were conducted with representative oomycete isolates. *P. cinnamomi*, *P. citrophthora*, and *Pythium ultimum* were all pathogenic in *J. regia*. Nevertheless, only *P. cinnamomi* and *P. citrophthora* were pathogenic to English walnut. *Py. ultimum* caused limited levels of root damage to English walnut seedlings. Our research indicates that as the Chilean walnut industry has expanded, so have walnut crown and root rots induced by oomycetes.

In the last 10 years, English walnut (*Juglans regia* L.) production area in Chile has expanded from 10,000 ha (2007) to 33,000 ha (2016), with 75,947 tons of walnuts produced in 2015 (ODEPA 2016). Chile is now the third-ranked exporter of walnuts worldwide. Chilean walnut production extends from the Atacama region in the north (latitude -27.36) to the Los Rios region in the south (latitude -39.80) and encompasses a wide range of climatic and edaphic conditions. English walnut orchards in all of these regions have reported trees with chlorosis, defoliation, shoot dieback, and root rot, often in association with crown rot. However, no recent surveys of this decline or its associated pathogens are available. Most commercial English walnut cultivars in Chile are grafted on English walnut rootstocks, which are considered to be highly susceptible to *Phytophthora* species (Mircetich and Matheron 1983).

In California, more than 10 species of *Phytophthora* have been associated with crown and root rots of walnut trees, with *P. cinnamomi* and *P. citricola* being the most aggressive and difficult *Phytophthora* species to control (Browne et al. 2011; Mircetich and Matheron 1983). In Europe, Belisario et al. (2001) reported that the main species affecting walnut is *P. cinnamomi*, but *P. cactorum*, *P. cambivora*, *P. chlamydospora*, *P. cryptogea*, *P. citricola*, *P. gonapodyides*, *P. megasperma*, and *P. nicotianae* were also involved (Belisario et al. 1997, 2001, 2003, 2006, 2012, 2016; Dervis 2016; Erwin and Ribeiro 1996; Kovacs et al. 2013; Kurbetli 2013; Vettraino et al. 2002, 2003). The first report of a *Phytophthora* species in English walnut orchards in Chile was made by English et al. (1967), who isolated *P. cactorum* from soil around declining English walnut trees in the Valparaíso region. At that time, English walnut orchards were

concentrated in the Metropolitana and O'Higgins regions, although there were no reports of walnut root and crown rot there. A few years later, *Phytophthora* species associated with root and crown rot of English walnut were reported in the Valparaíso region, including *P. cactorum* (INIA 1969), *P. cinnamomi*, and *P. citrophthora* (Rojic and Cancino 1975). Since the 1970s, the Chilean English walnut industry has been expanding, with a strong increase in the last 10 years. In a 2015–2016 survey conducted in central Chile, *P. cinnamomi* was found in 20 of the 49 English walnut orchards (Guajardo et al. 2017). This pathogen has also been isolated from grapes (*Vitis vinifera*) (Latorre et al. 1997), avocado (*Persea americana*) (Besoain et al. 2005), and blueberry (*Vaccinium corymbosum*) (Larach et al. 2009) in central Chile.

Pythium (*Py.*) and *Phytophythium* (*Phy.*) species have been reported to cause root rot in many perennial crop plants, including apple (Utkhed and Smith 1991), grape (Spies et al. 2011), and pistachio (Fichtner et al. 2016).

It may be possible to reduce losses due to root and crown rot diseases among Chilean English walnut orchards by implementing integrated pest management approaches that include the use of resistant rootstocks, cultural management practices, and the use of systemic fungicides (Browne et al. 2011, 2015; Mircetich et al. 1998). However, implementation of such approaches would be greatly facilitated by knowledge of the pathogens, their geographical distributions, and their biology in the regions of concern. For example, rootstocks of *J. regia* are more susceptible than Paradox hybrid rootstock (*J. regia* × *J. hindsii*) to several species of *Phytophthora* (Matheron and Mircetich 1985; Mircetich et al. 1998) that attack walnut in California, but it is unknown whether root and crown rot pathogens in Chile are similar to those obtained in California, so we cannot conclude that they have an equal level of susceptibility. Therefore, this study was conducted to: (i) evaluate the incidence and severity of root and crown rot disease in English walnut trees in central Chile, (ii) isolate and identify oomycetes that cause root and crown rot of English walnut in Chile, and (iii) study the pathogenicity of oomycetes commonly associated with root and crown rot in English walnut.

Materials and Methods

Field survey. In the spring (October to December) of 2015, an extensive diagnostic survey was conducted in 45 English walnut orchards distributed among the five main English walnut producing regions of Chile: Coquimbo, Valparaíso, Metropolitana, O'Higgins,

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and Maule. In each region, nine randomly selected English walnut orchards, ranging from 1 to 21 years old, were surveyed (Fig. 1). All English walnut trees were grafted on *J. regia* rootstock. The proportion of orchards with at least one tree affected by root and crown rot was estimated. To determine the incidence and severity of this disease, quadrants of 100 trees were randomly selected and inspected for root and crown rot disease symptoms: leaf chlorosis, leaf fall, and crown canker. Incidence was estimated as the percentage of diseased trees over the total number of trees evaluated ($n = 100$). Disease severity was determined using a scale of 0 to 4, where: 0 = a healthy tree, 1 = a tree with green leaves, but with irregular sprouting, 2 = a tree with irregular sprouting and defoliated zones, 3 = a tree with chlorosis, irregular bud breaking, and defoliated zones, and 4 = a defoliated and dead tree. The severity of disease in each orchard was expressed using the canopy damage index (cDI) (McKinney 1923), according to the following equation: $cDI (\%) = (\sum(nv) / (NV)^{-1} 100)$, where n = number of trees per disease rating scale; v = disease rating scale; N = total number of trees observed; V = maximum range of the attack scale.

Sampling and isolation. From October 2015 to April 2017, rotten root samples and occasionally tissue samples taken from the margins of trunk cankers were collected from the 45 orchards previously surveyed. For additional comparisons, samples were taken from 10 orchards in southern Chile (Bío Bío Region). Soil samples were taken 5 to 30 cm deep where the main feeder roots were located. A total of 140 samples were taken to the laboratory and used for isolations (Guajardo et al. 2017). Necrotic tissue samples (3 to 4 mm long), obtained from the margin of the lesions of root rot cankers, were washed three times with sterile distilled water (SDW) and plated

on P₅ARP semiselective medium (5 mg pimarin, 250 mg ampicillin, 10 mg rifampicin, 100 mg pentachloronitrobenzene, and 17 g cornmeal agar in 1,000 ml distilled water) and potato-dextrose agar (PDA; Merck, Darmstadt, Germany) acidified with 1 ml of lactic acid (APDA) for cankered tissue isolation (Jeffers and Martin 1986). Plates were incubated in the dark for 7 days at 25°C. Soil samples (300 g) were placed in a 500-ml pot and saturated with SDW before placing on *Rhododendron* leaves as bait (Themann and Werres 1998). After 96–120 h, the leaves exhibiting necrotic lesions were cut (5 mm pieces) and cultured in P₅ARP medium. Hyphal tips were subcultured from the margins of colonies developed on P₅ARP and transferred to fresh APDA and cornmeal agar (CMA; Merck, Darmstadt, Germany).

Morphological and molecular identification. *Phytophthora* isolates were identified by comparing morphological characteristics of the colonies, sporangia, chlamydospores, and hyphal swellings, with descriptions reported in the literature (Erwin and Ribeiro 1996; Stamps et al. 1990). For *Pythium* species we used the descriptions of Waterhouse (1968) and De Cock et al. (2015). *Phytophthora*-like isolates were identified molecularly by the use of the ITS region and confirmed by beta-tubulin gene. Analysis of sexual structures was not included. Colony morphology was determined on 5-day-old cultures growing on APDA and CMA at 25°C in the dark. Sporangium production was induced by placing mycelial discs (3 mm diameter) in carrot juice (500 g carrot boiled for 15 min in 1 liter SDW) on glass plates under continuous light for 48 h at 24°C (Larach et al. 2009). Colonies growing from the mycelial discs were washed with SDW and treated for 3 min at 5°C soaking in saline solution (2.36 g CaNO₃, 0.5 g KNO₃, 1 g MgSO₄, and 1 ml chelated iron solution [13 g·liter⁻¹ ethylene diamine tetra acetic iron, 7.5 g·liter⁻¹ KOH, and 24.5 g·liter⁻¹ FeSO₄] in 1 liter of SDW) (Erwin and Ribeiro 1996). Subsequently, the mycelium was rinsed with SDW and incubated in 1% wt/vol soil extract for 48 h at 24°C under light. Sporangia, chlamydospores, and hyphal swellings were then examined under a light microscope. In total, 30 samples of each structure were examined per isolate (Nikon, eclipse Ni-U).

At least one isolate tentatively identified as *Phytophthora* in each English walnut production region was confirmed by molecular analyses. DNA was extracted from fresh mycelium developed in pure cultures on PDA medium for 48–72 h at 25°C using the DNeasy Plant Mini Kit (QIAGEN). The ITS1-5.8S-ITS2 region of the ribosomal DNA was amplified using the primers ITS4 5'-TCCTCCGCTTATTGATATGC-3' and ITS5 5'-GGAAGTAAAGTCGTAACAAGG-3' (White et al. 1990). The beta tubulin gene was amplified using the primers BT2aF 5'-GGTAACCAATCGGTGCTGCTTTC-3' and BT2bR 5'-ACCCTCAGTGTAGTGACCCTTGGC-3' (Glass and Donaldson 1995). Amplification reactions were prepared in the final volume of 22 µl. All the employed methodology was done according to Sapphire Amp Fast PCR Master Mix (Takara, Japan). For each reaction, 1 µl of DNA was used. All reactions were performed using a thermal cycler (Bioer, China) under the following conditions: 94°C, 1 min initial denaturation, followed by 35 cycles that included denaturation at 98°C for 5 s, annealing at 55°C for 5 s, extension at 72°C for 5 s, and a final elongation at 72°C for 1 min. The PCR products were visualized by electrophoresis in 1% agarose in TAE buffer (Winkler, Chile) stained with gel red (Biotium, USA) and visualized on a UV transilluminator (Vilber Lourmat, France). Amplified products were purified and sequence in both directions by Macrogen (South Korea). Analyses of the sequence data were conducted using the Geneious 10.0.6 software and aligned using BLASTN (NCBI/BLAST), considering the searches in GenBank that showed 99 to 100% similarity with reference sequences, including ex-types. Sequences were deposited in GenBank (Table 1).

Pathogenicity test. Pure cultures of oomycete species were used for pathogenicity tests. The tested isolates included three isolates of *P. cinnamomi* (PN 1858, PN 1955, and PN 1956), two isolates of *P. citrophthora* (PN 1994 and PN 1995), two isolates of *P. humicola* (PN 1875 and PN 1943), three isolates of *Phy. vexans* (PN 1866, PN 1872, and PN 1909), and one isolate of *Py. ultimum*

Fig. 1. English walnut orchards in Chile covered in diagnostic surveys for root rot disease, carried out in the spring of 2015.

(PN 1665). Considering that *Phy. litorale* and *Phy. mercuriale* were only occasionally isolated, no pathogenicity tests were performed with isolates of these species. Three-month-old ungrafted plants of *J. regia*, grown in 3-liter pots with 1:1 peat:sterile sand substrate, were used for inoculation. The plants were watered every 2 days and fertilized with 9 g Ultrasol Multipurpose (SQM, Santiago, Chile) per plant, applied once at preplanting. Four plants for each isolate were used, and four plants remained noninoculated as controls. Plants were inoculated with zoospores obtained from a suspension of sporangia (mycelia was filtered with a sterile gauze) incubated for 20 min at 4°C and then 1 h at 25°C, until the zoospores were released (Messenger et al. 2000). A drop of the zoospore suspension was mounted in Lactophenol blue solution (Merck, Darmstadt, Germany) and counted with the aid of a Neubauer chamber and a microscope at 20× magnification. Zoospore concentrations were adjusted with SDW. Unwounded plants were inoculated with 50 ml of SDW containing 1×10^5 zoospores ml⁻¹ and the controls treated with 50 ml SDW. Soil was saturated for 48 h and plants were maintained in a greenhouse at 25 ± 5°C until symptoms were obtained or for a maximum of 2 months. If plants remained symptomless for 2 months, the inoculation was repeated using fresh wounded plants that were inoculated with a 5-mm mycelial plug inserted into the stem. Control plants were also wounded, but only a sterile APDA plug was used. Root necrosis, percentage of fallen leaves, and presence of cankers were measured after 2 months. Root damage, measured as the presence of necrotic root lesions, was evaluated using a scale previously described (Vettraino et al. 2003), where: 0 = no damage; 1 = up to 25% damage;

2 = 25 to 50% damage; 3 = 50 to 75% damage; 4 = 75 to 100% damage. Pathogenicity tests were repeated.

Statistical analysis. To analyze differences in the percentage of fallen leaves obtained among treatments ($n = 4$ for *P. cinnamomi* and *Phy. vexans*, $n = 3$ for *P. citrophthora* and *P. humicola*, $n = 2$ for *Py. ultimum*; 4 replicates for each treatment) an analysis of variance (ANOVA) was performed following a complete randomized design. Percent data were square root transformed before analysis. The means were separated using a Fisher's LSD test ($\alpha = 0.05$). The root

Table 3. Oomycete species isolated from decaying English walnut trees in the main walnut-production regions of Chile

Oomycetes	Orchards positive to oomycete ^a						
	Coq	Val	Met	OHi	Mau	Bio	Total
<i>Phytophthora cinnamomi</i>	2	8	1	6	4	4	25
<i>Phytophthora citrophthora</i>	0	1	0	0	0	0	1
<i>Phytophthora humicola</i>	1	0	0	0	1	0	2
<i>Phytopythium vexans</i>	3	3	4	1	0	0	11
<i>Phytopythium litorale</i>	1	1	0	0	0	1	3
<i>Phytopythium mercuriale</i>	0	0	0	0	1	0	1
<i>Pythium ultimum</i>	1	2	0	0	0	0	3
<i>Pythium</i> sp.	3	1	2	2	2	0	10
Total orchard sampled, no.	10	13	9	9	10	4	55

^a The administrative regions of Chile were: Coq: Coquimbo region, Val: Valparaíso region, Met: Metropolitana region, OHi: O'Higgins region, Mau: Maule region, Bio: Bío Bío region.

Table 1. Oomycete species isolated from English walnuts in Chile identified morphologically and molecularly in this study

Collection number	Location ^a	Identity (%) ITS-Btub	Accession number ITS/Btub	GenBank ID ITS/Btub
<i>Phytophthora cinnamomi</i>				
PN 1859	Coquimbo	99-99	KU723593.1/KU899233.1	MH424500/MH427876
PN 1858	Valparaíso	99-99	KU723593.1/KU899233.1	KU961897/MH427875
PN 1886	Metropolitan	99-99	HQ643400.2/AB856781.1	KU961899/MH427900
PN 1959	O'Higgins	99-99	KU723593.1/KU899233.1	MH424502/MH427886
PN 1874	Maule	99-99	GU799635.1/KU899233.1	KU961907/MH427879
PN 1980	Bío Bío	100-99	KU723593.1/HM041802.1	MH236248/MH427889
<i>Phytophthora citrophthora</i>				
PN 1994	Valparaíso	100-98	KT148901.1/JN605845.1	MH368449/MH427892
<i>Phytophthora humicola</i>				
PN 1875	Maule	99-99	JQ757060.1/JN935975.1	KU961908/MH427893
PN 1943	Coquimbo	98-II ^b	JQ757060.1/Na ^c	MH368445/Na ^c
<i>Phytophythium litorale</i>				
PN 1868	Coquimbo	99-II ^b	KT148891.1/Na ^c	KU961901/Na ^c
PN 1857	Valparaíso	98-II ^b	HQ643386.2/Na ^c	KU961896/Na ^c
PN 1982	Bío Bío	100-II ^b	KT148891.1/Na ^c	MH368448/Na ^c
<i>Phytophythium mercuriale</i>				
PN 1957	Maule	99-II ^b	KU211357.1/Na ^c	MH368446/Na ^c
<i>Phytophythium vexans</i>				
PN 1866	Coquimbo	99-99	HQ643400.2/AB856781.1	KU961899/MH427900

^a Administrative regions of Chile.

^b II: Insufficient information to analyze.

^c Na: Not analyzed.

Table 2. Survey of root and crown rot disease in English walnut orchards in central Chile

Region ^a	Affected orchards (%)	Mean incidence ^b (%)	Mean severity ^c (%)
Coquimbo	88.9	12.44	6.75
Valparaíso	100.0	28.56	18.08
Metropolitana	88.9	8.44	5.25
O'Higgins	88.9	18.33	13.81
Maule	100.0	7.89	6.75
Total mean	93.3	15.5	10.3

^a Administrative regions of Chile.

^b The incidence was determined in 100 trees per orchard.

^c The severity was estimated according to a canopy damage index cDI (%): $\sum(nv) (NV)^{-1} 100$.

damage was statistically analyzed with the Wilcoxon test for non-parametric comparison ($\alpha = 0.05$). Data were analyzed using JMP v.13.0 software (SAS Institute Inc., Carolina del Norte, USA).

Results

Disease incidence and severity. Chlorosis, small leaves, defoliation, and shoot dieback were observed in 42 (93.3%) of the 45 surveyed orchards, in all the walnut growing regions (Table 2). Brown to black cankers on the root and collar area of the trunk was found in 17 (37.8%) of the 45 surveyed orchards. Dead trees were occasionally observed. The mean incidence and mean severity varied from 7.9 to 28.6% and from 5.3 to 18.1%, respectively.

Isolation and identification. A total of seven oomycete species were identified from rotten roots and soil samples. Oomycete isolations from trunk pieces collected from cankered tissues were unsuccessful. Based on morphological and molecular identification, the oomycete species identified were *P. cinnamomi*, *P. citrophthora*, *P. humicola*, *Phy. vexans*, *Phy. litorale*, *Phy. mercuriale*, *Py. ultimum*, and *Pythium* sp. (Table 3). All the isolates had hyaline, cenocytic hyphae on APDA media. Sporangia were produced by all isolates (Erwin and Ribeiro 1996) (Table 4; Supplementary Fig. S1).

P. cinnamomi colonies were stellate or rosaceous; they produced hyphal swellings and chlamydospores. Sporangia were nonpapillated, ellipsoid to ovoid, and internal proliferations were obtained. The two isolates of *P. humicola* had dense, cottony colonies. Hyphal swellings and chlamydospores were not observed. Sporangia were papillated with internal proliferations. Isolates of *P. citrophthora* had a stellate colony on APDA, hyphal swellings were not produced, and chlamydospores were obtained. Sporangia were variable in shape with one or two papillae. Internal proliferations were absent. Isolates of *Phy. vexans* produced rosaceous colonies on APDA. Hyphal swellings and chlamydospores were not observed. Sporangia were subglobose, broad ovoid or obpyriform, nonpapillated, and/or proliferations were not observed. Isolates of *Phy. litorale* produced a dense, cottony colony on APDA. Hyphal swellings or chlamydospores were absent. Sporangia were subglobose to ovoid, usually with one papilla and internal proliferations. Isolates of *Phy. mercuriale* had cottony colonies on APDA. Hyphal swellings or chlamydospores were not observed. Sporangia were papillated subglobose to ob-ovoid. Isolates of *Py. ultimum* had stellate colonies on APDA. No hyphal swellings or chlamydospores were observed. Sporangia were spherical, nonpapillated, and proliferations were not produced (Table 4).

The most frequently isolated *Phytophthora* species was *P. cinnamomi*, recovered from 45.5% of the orchards, followed by *P. humicola* in 3.6% and *P. citrophthora* in 1.8% of the orchards. Among *Phytophythium*, *Phy. vexans* was recovered from 20% of the orchards, followed by *Phy. litorale* in 5.5% of the orchards and *Phy. mercuriale* in 1.8% of the orchards. *Phytophthora cinnamomi* was

recovered from all production regions and *Phy. vexans* was recovered in samples from the Coquimbo, Valparaíso, Metropolitana, and O'Higgins regions (Table 3).

Molecular results. The molecular analysis corroborated the morphological approach. The species isolated from English walnut orchards were identified by ITS and beta tubulin genes as: *P. cinnamomi*, *P. citrophthora*, *P. humicola*, *Phy. litorale*, *Phy. mercuriale*, and *Phy. vexans* (Table 1; Supplementary Table S1 and S2). All isolates were identified, with an identity percentage between 98 and 100% for both gene regions.

Pathogenicity tests. Isolates of *P. cinnamomi* and *P. citrophthora* were pathogenic, causing crown and root rot in *J. regia* plants (Table 5). However, *P. humicola* isolates were nonpathogenic in

Table 5. Pathogenicity of isolates of *Phytophthora* (*P.*), *Phytophythium* (*Phy.*), and *Pythium* (*Py.*) species determined in 1-year-old English walnuts

Isolates	Defoliation (%)	Cankered plants, no. ^a	Mean of root damage index (_{RDI})
<i>P. cinnamomi</i>			
Noninoculated control	0	0/4	0
PN 1858	81 ^b	4/4	4 ^c
PN 1955	61 ^b	4/4	4 ^c
PN 1956	31 ^b	4/4	4 ^c
<i>P. citrophthora</i>			
Noninoculated control	0	0/4	0
PN 1994	27 ^b	3/4	4 ^c
PN 1995	27 ^b	3/4	3,5 ^c
<i>P. humicola</i>			
Noninoculated control	0	0/4	0
PN 1875	0	0/4	0
PN 1943	0	0/4	0
<i>Phy. vexans</i>			
Noninoculated control	0	0/4	0
PN 1866	0	0/4	0
PN 1872	0	0/4	0
PN 1909	0	0/4	0
<i>Py. ultimum</i>			
Noninoculated control	0	0/4	0
PN 1665	0	0/4	1 ^c

^a Numerator means number of plants with canker, denominator means total of plants evaluated.

^b Number of fallen leaves: values are significantly different from the control ($\alpha = 0.05$) using Fisher's least significant difference (LSD) test.

^c Mean of root damage index: values are significantly different from the control ($\alpha = 0.05$) using non parametric Wilcoxon test.

Table 4. Morphological characteristics of sporangium of oomycete species isolated in the current study

	Shape	Papillae	Proliferation	Mean length (μm)	Mean width (μm)	Mean length/width ratio
<i>Phytophthora</i>						
<i>P. cinnamomi</i>	Ellipsoid to ovoid	Non	Internal	56.0 ± 12.3 ^a	34.8 ± 8.1 ^a	1.6 ± 0.2 ^a
<i>P. citrophthora</i>	Spherical, ovoid, obpyriform to ellipsoid	Present, 1 or 2	ND ^b	43.8 ± 4.3	29.0 ± 3.1	1.5 ± 0.1
<i>P. humicola</i>	Ovoid to obpyriform	Non	Internal	60.5 ± 8.8	47.8 ± 9.4	1.3 ± 0.1
<i>Phytophythium</i>						
<i>Phy. vexans</i>	Subglobose, broad ovoid or obpyriform	Non	ND	24.8 ± 3.7	19.9 ± 2.6	1.3 ± 0.2
<i>Phy. litorale</i>	Subglobose or ovoid	Usually present	Internal	30.4 ± 1.6	25 ± 1.2	1.2 ± 0.0
<i>Phy. mercuriale</i>	Subglobose to ovoid	Present	ND	53.2 ± 8.6	22.8 ± 1.2	2.3 ± 0.3
<i>Pythium</i>						
<i>Py. ultimum</i>	Spherical	Non	ND	22.6 ± 3.0	19.4 ± 2.0	1.2 ± 0.1

^a ± SD: Standard deviation.

^b ND: Not detected.

wounded and unwounded plants. *Py. ultimum* only caused leaf chlorosis and slight root rot (Table 5).

Defoliation, crown rot, and root damage were observed in plants inoculated with *P. cinnamomi* 7 days after inoculation (Table 5, Fig. 2e). Root damage was observed as a cortical black necrosis, but the xylem was not affected (unpublished results). Crown rot was characterized by a cortical necrosis from 0.3 to 4.8 cm long, extending from the cortex to the cambial tissue and, in most plants, exudation of black droplets was also observed (Fig. 2d). On the other hand, *P. citrophthora* caused cortical necrosis from 0.5 to 7.6 cm long 14 days after inoculation (Table 5). Crown rot always progresses from the cambial zone to the surface of the shoots. Plants inoculated with *Py. ultimum* did not lose leaves or develop crown rot; however, one isolate produces a slight root necrosis 45 days after inoculation. Pathogens from all trials were reisolated from root injuries and were morphologically identified. No pathogens were isolated from asymptomatic control plants. Plants inoculated with *P. humicola* and *Phy. vexans* showed no symptoms 2 months after zoospore inoculation, and no symptoms were observed 2 months after wounding the main stem and placing mycelium of *P. humicola* and *Phy. vexans*.⁷

Discussion

This study provided the first quantitative report of the incidence and severity of Phytophthora root and crown rot disease of English walnut in Chile, demonstrating the presence of *P. cinnamomi*, *P. citrophthora*, *P. humicola*, *Phy. vexans*, *Phy. litorale*, *Phy. mercuriale*, *Py. ultimum*, and *Pythium* sp. Nevertheless, only *P. cinnamomi* and *P. citrophthora* were pathogenic to English walnut. *Py. ultimum* caused limited levels of root damage to English walnut seedlings, but defoliation or crown rot cankers were not obtained. *Py. ultimum* has been described as a ubiquitous soil inhabitant that can attack many horticultural crops (Hendrix and Campbell 1973). To our knowledge, this is the first study that has completed Koch's postulates for *Py. ultimum* in *J. regia* worldwide. However, further studies are needed before concluding that *Py. ultimum* is the cause of root and crown rot of English walnut. *Phy. vexans* has been described as a pathogen in several species such as Fraser fir and apple seedlings (Ivors et al. 2008; Tewoldemedhin et al. 2011), and it was widely distributed in Chilean English walnut orchards; nevertheless, this species was not pathogenic to *J. regia* plants.

This study also demonstrated the importance of Phytophthora root and crown rot affecting English walnut at a national level. In previous studies (English et al. 1967; INIA 1969; Rojic and Cancino 1975), the disease was reported only in the Valparaíso region of Chile; however, the present survey documented the presence of a high incidence of Phytophthora root and crown rot in all English walnut along a 700-km north-south axis, across a range of diverse climatic zones and diverse soil conditions, with mean annual rainfall of 370 to 1,055 mm. However, in the present study, the highest incidence of the disease was observed in the Valparaíso and O'Higgins regions, where the oldest English walnut orchards were planted, and where several avocado orchards were converted to walnut, even after avocado root rot disease had been previously detected (S. Saa, unpublished).

Isolation of oomycete species was not successful in all orchards where symptoms were observed. This could be due to the fact that in several orchards the trees were already dead and saprophytic species had colonized the tissues before sampling, or to the possible presence of inhibitory substances secreted by the walnuts in response to injuries made while selecting the tissue samples for isolations, or perhaps isolations were performed when *Phytophthora* was less active in the orchards. For example, in Rojic and Cancino (1975), the isolation of *Phytophthora* from English walnut trees was successful between April and May, which corresponds to autumn in Chile. Mircetich and Matheron (1983) agree that the success of isolation from California walnut orchards was greater from late autumn to early spring, and was sporadic and difficult from late spring and summer. In our case, isolations were more successful in early spring and autumn than in summer months.

Morphological and molecular features allowed the identification of *P. cinnamomi* and *P. citrophthora* attacking English walnuts trees in Chile, coinciding with previous reports in Chile and in the world (Rojic and Cancino 1975; Stamps et al. 1990). In previous studies, *P. cactorum* was also identified affecting English walnut trees in the Valparaíso region (English et al. 1967; INIA 1969). However, in this study *P. cactorum* was not isolated. This species has also been reported to cause crown rot of walnut trees in California (Mircetich and Matheron 1983), so it is plausible that this species could be found in other Chilean English walnut orchards not surveyed in the present study.

Our pathogenicity tests revealed that *P. cinnamomi* and *P. citrophthora* were the most aggressive species affecting *J. regia* plants. The result of the *P. cinnamomi* pathogenicity test is in accordance with previous studies carried out in Europe (Belisario et al. 2006, 2009) and the United States (Mircetich and Matheron 1983). *P. citrophthora* has also been described affecting English walnuts in the United States and Greece (Holevas et al. 2000; Mircetich and Matheron 1983).

Because our research indicates that *P. cinnamomi* is the most widely distributed species affecting English walnut in Chile, integrated management approaches for the disease should emphasize this species. It may be advisable to avoid planting English walnuts in soils previously devoted to avocado or blueberry production that sustained losses due to the pathogen. However, because the RX1 rootstock (*Juglans microcarpa* × *J. regia*) was recently reported to provide superior resistance to *P. cinnamomi* in California (Browne et al. 2015), this rootstock and others with similar traits would be promising candidates for testing in Chilean orchards infested with the pathogen. Fosetyl-al has been effective for *P. cinnamomi* control (Belisario

Fig. 2. *Phytophthora cinnamomi* root rot in English walnut (*Juglans regia*). (a) English walnut tree with chlorosis and leaf fall. (b) Crown rot in an English walnut tree. (c) Pathogenicity test on *J. regia* with three isolates of *P. cinnamomi*. (d) Cortical necrosis and exudation of black droplets. (e) Rooted roots.

et al. 2009). Phosphonate treatments provided some suppression of *P. citricola* on walnut (Browne et al. 2011); however, future research in Chile may be needed to determine its effectiveness against *P. cinnamomi* in English walnut.

Finally, in addition to a focus on management of *P. cinnamomi*, it will also be important to monitor and manage, over time, additional oomycete pathogens of English walnut in Chile. For example, we found that *P. citrophthora* was pathogenic in *J. regia* plants, and therefore rootstock resistance, as well as chemical and cultural control strategies for this species, should also be evaluated under Chilean conditions.

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