BIOLOGY AND MANAGEMENT OF PHYTOPHTHORA CROWN AND ROOT ROT OF WALNUT

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ABSTRACT

Our objectives in 2008 were to: 1) evaluate elite Paradox hybrid clones for resistance to *Phytophthora cinnamomi and P. citricola* and 2) examine contributions of pathogens to decline of English walnut orchards on Paradox rootstock. Two greenhouse evaluations of resistance to *P. cinnamomi* and *P. citricola* were completed in 2008, one involving 17 and another involving 14 hybrid walnut rootstock clones. The clones were selected previously for putative resistance to *Phytophthora* or other pathogens or genetic backgrounds of interest. In each trial with *P. cinnamomi* and *P. citricola*, seedlings of Northern California black walnut (*Juglans hindsii*) and Chinese wingnut (*Pterocarya stenoptera*) were used as highly susceptible and highly resistant standards, respectively. Depending on the clone, the maternal parents of the hybrids were *J. californica* (clones AX1, AX2, AX3), *J. hindsii* (GZ2, GZ3, J1D, UZ1, UZ2, Vlach, VX211, and XZ1), *J. microcarpa* (RX1), *J. nigra* (CW1, MW1), *J. californica* x *J. nigra* (UX2), and (*J. major x hindsii*) x *nigra* (AZ025, JZ1); the paternal parent for each clone was *J. regia*. In both trials, Northern California black walnut and Chinese wingnut were highly susceptible and highly resistant, respectively, to both species of *Phytophthora*. All three of the AX clones were relatively susceptible to both pathogens in both trials; this is consistent with previous results and indicates that *J. californica* as a sole maternal parent in hybrids with *J. regia* can lead to susceptibility to *Phytophthora*. Conversely, the *microcarpa* hybrid was relatively resistant to both pathogens in both trials, confirming its responses in previous years and suggesting that *J. microcarpa* should be explored further for contributions of resistance to *Phytophthora* and other pathogens. The other hybrid clones were moderately susceptible to moderately resistant to *P. cinnamomi* and *P. citricola*, depending upon the trial and clone, but all of them sustained moderate to severe levels of crown and/or root rot in at least one trial with at least one of the pathogens. For objective 2, we examined walnut trees exhibiting decline and death on Paradox rootstock in Butte, San Joaquin, Merced, and Kings Counties and used culture- and PCR-based detection methods for fungi, oomycetes, and bacteria. Additional trees on Northern California black walnut rootstock were sampled in Kings County. The declining trees on Paradox rootstock in Butte County apparently were suffering from waterlogging and resulting secondary decay of roots. *Phytophthora* spp. were implicated in crown and root rot on Paradox rootstock on two trees in Merced County and several trees on Northern California black rootstock in Kings County. In contrast, no pathogen or environmental factor was linked by surveys or diagnostics to basal trunk and root crown cankers on 16 trees on Paradox rootstock in Kings County; only non-pathogenic organisms were detected (principally saprophytic bacteria). Furthermore, PCR primers reported to be semi-selective for fungi and oomycetes were not selective against genomic walnut DNA. We are broadening the approach to determining etiology of the Paradox crown rot by: using suppression selective hybridization to isolate DNA associated with the disease, intensifying sampling during periods when cankers are most active (i.e., mid-summer), and considering possible contributions of herbicides to the disease (i.e., glyphosate, paraquat).

INTRODUCTION

Crown and root rots caused by species of *Phytophthora* are among the most serious diseases of English walnut trees worldwide. In California, more than 10 species of *Phytophthora* have been implicated in the diseases, but *P. cinnamomi* and *P. citricola* were determined to be the most aggressive. Northern California black walnut (NCB) and some selections of Paradox hybrid seedling rootstock are highly and moderately susceptible, respectively, to each of these pathogens. This project is proceeding under a hypothesis that the most effective and economical approach to managing Phytophthora crown and root rots and other soilborne diseases is to select and use rootstocks with tolerance or resistance to the pathogens.

We have worked with the Walnut Improvement Program (WIP), commercial nurseries, and Wes Hackett to evaluate diverse hybrid walnut rootstock clones for resistance to *Phytophthora*. The clones were micropropagated from seedlings selected for their putative resistance to *P. citricola* and other traits of interest. In 2007, we began screening clones for resistance to *P. cinnamomi* as well as to *P. citricola*, and this was continued in 2008.

Two of the clones, RX1 and VX211 have emerged as promising rootstocks. RX1 consistently has exhibited good resistance to *P. citricola*, and its initial expressions of resistance to *P. cinnamomi* in 2007 were encouraging. VX211 also has expressed resistance to *P. citricola*, and it is desirable for its high vigor and apparent tolerance to lesion nematode. Below we report on further evaluations of resistance to *Phytophthora* in RX1, VX211, and 15 additional hybrid clones.

Paradox rootstock is favored in the California walnut industry due to its vigor and superior resistance to most species of *Phytophthora*, but its widespread use has revealed weak points. Susceptibility to *Agrobacterium tumefaciens* is probably its most serious weakness, but field observations suggest that, under some conditions, Paradox also is more prone than NCB to waterlogging damage. In addition, we have observed crown rot on Paradox rootstock that appears to be distinct from waterlogging damage and has not been associated with either *Phytophthora* or *Armillaria*. Below, under objective 2, we report on our examinations of trees affected by the latter type of crown rot symptom.

OBJECTIVES

Objectives

1. Evaluate elite Paradox hybrid clones for resistance to *Phytophthora cinnamomi and P. citricola*.
2. Examine contributions of pathogens to decline of English walnut orchards on Paradox rootstock.

PROCEDURES

Objective 1. Evaluations of resistance to *P. citricola* in hybrid clones.

Two greenhouse evaluations of resistance to *P. cinnamomi* and *P. citricola* were completed in 2008, one involving 17 and another involving 14 hybrid walnut rootstock clones. The clones were selected previously for putative resistance to *Phytophthora* or other pathogens or genetic backgrounds of interest. In each trial with *P. cinnamomi* and *P. citricola*, seedlings of Northern California black walnut (*Juglans hindsii*) (NCB) and Chinese wingnut (*Pterocarya stenoptera*) were used as highly susceptible and highly resistant standards, respectively. Depending on the clone, the maternal parents of the hybrids were *J. californica* (clones AX1, AX2, AX3), *J. hindsii* (GZ2, GZ3, J1D, UZ1, UZ2, Vlach, VX211, and XZ1), *J. microcarpa* (RX1), *J. nigra* (CW1, MW1), *J. californica* x *J. nigra* (UX2), and (*J. major x hindsii*) x *nigra* (AZ025, JZ1); the paternal parent for each clone was *J. regia*.

After rooting and greenhouse acclimatization, plants to be used for evaluations of resistance were subjected to several months of chilling at 6 °C and transplanted and grown in 1-liter pots in a greenhouse. The plants were kept trimmed to a height of about 1 ft. to equalize and maintain their size.

In May 2008 (experiment 1) and again in July 2008 (experiment 2), individual plants from the 1-liter pots were transplanted into 2-liter pots filled with UC potting mix soil that was either artificially infested with *P. citricola* or *P. cinnamomi* (45 ml of V8 juice-oat-vermiculite substrate infested with one of the pathogens per liter of the potting mix) or treated as a control (45 ml sterile substrate per liter of potting mix). In each experiment, there were 5 replicate plants in pots of non-infested soil and 10 to 20 replicate plants in infested soil in a split-plot design (main plots were inoculum treatments, subplots were rootstocks) among 5 blocks. Every 2 weeks after transplanting the soil in each pot was flooded for 48 h. Three months after transplanting, the root systems were washed free from soil and evaluated visually for incidence and severity of crown and root rot.

Objective 2. Examining contributions of pathogens to decline of English walnut orchards on Paradox rootstock.

Decline associated with crown rot on Paradox rootstock. Twenty-one trees of English walnut on Paradox rootstock exhibiting basal trunk cankers and crown rot were sampled between 2 May and 10 October 2008 and subjected to diagnostic assays. Sixteen of the trees were in six different orchards in Kings County (age approx. 10 to 20 yr), and five of the trees were in two orchards in Merced County (age approx. 8 years and 30 yr). Necrotic bark was removed from the margins of the cankers, and soil was collected around the diseased trunks. The bark samples were cut into pieces approximately 2 to 3 mm across. A subsample of the pieces was prepared for culturing by either rinsing in sterile water or bleaching in 0.6% sodium hypochlorite (pH 7.1), followed by rinsing in sterile water for 1 min. The free water was blotted away on tissue paper and the necrotic bark pieces were cultured on the following media: corn meal agar amended with pimaricin, ampicillin, rifampicin, and PCNB (PARP, semi-selective for oomycetes, including *Phytophthora* species); water agar amended with ampicillin (for isolation of true fungi); and, for

some samples, potato dextrose agar amended with ampicillin, and malt agar. Additional subsamples of the rinsed and bleached necrotic bark tissues were ground in sterile water using a mortar and pestle and used for bacterial dilution plating on 10% tryptic soy broth agar and additional media for isolation of bacteria. Finally, remaining subsamples of the necrotic bark pieces were frozen and used for subsequent DNA extractions and amplifications using semi-selective PCR primers for fungi, oomycetes, and bacteria (Table 1). The soil samples were subjected to baiting assays with pear fruits to detect species of *Phytophthora*.

Decline associated with other symptoms. In addition to the diagnostics for trees on Paradox rootstock affected by crown and trunk cankers as described above, 15 English trees on Paradox rootstock affected by root rot but lacking distinct crown and trunk cankers were sampled from Butte, San Joaquin, and Kings Counties. Also, three dying English walnut trees with crown rot on NCB walnut rootstock were sampled in Kings County. Root segments or crown tissues from each of these orchards were cultured on PARP medium, and soil samples collected around the trees were baited with pear fruits to detect *Phytophthora* spp.

RESULTS AND DISCUSSION

Objective 1. Evaluations of resistance to *P. cinnamomi* and *P. citricola* in hybrid clones.

In both greenhouse experiments evaluating resistance to *P. cinnamomi* and *P. citricola,* Northern California black walnut and Chinese wingnut were highly susceptible and highly resistant, respectively, to both species of *Phytophthora* (Figs. 1,2). All three of the AX clones were relatively susceptible to both pathogens in both trials; this is consistent with previous results and indicates that use of *J. californica* as a sole maternal parent in hybrids with *J. regia* can lead to susceptibility to *Phytophthora*. Conversely, the *microcarpa* hybrid was relatively resistant to both pathogens in both trials, confirming its responses in previous years and suggesting that *J. microcarpa* should be explored further for contributions of resistance to *Phytophthora* and other pathogens. The other hybrid clones were moderately susceptible to moderately resistant to *P. cinnamomi* and *P. citricola,* depending upon the trial and clone, but all of them sustained moderate to severe levels of crown and/or root rot in at least one trial with one of the pathogens.

For several selections, crown and/or root rot was less severe in the experiment started in May 2008 (Fig. 1) than that started in July 2008 (Fig. 2). It is possible that warmer temperatures in the greenhouse in July suppressed disease development.

We intend to continue evaluations of resistance to *P. cinnamomi* and *P. citricola* in new and existing walnut rootstock hybrid clones, especially in genetic backgrounds that have offered promising traits. In collaboration with other labs, a broader examination of *J. microcarpa* x *J. regia* hybrids is being pursued. Also, hybrids of *J. ailantifolia* x *J. regia* will be tested for resistance to *Phytophthora* spp.

Objective 2. Determining etiology of the crown and root rot on Paradox hybrid rootstock.

Decline associated with crown rot on Paradox rootstock. With little exception, no known pathogens were detected from cankers on Paradox rootstock in Kings and Merced Counties.

Phytophthora cryptogea/*drechsleri* was isolated from the roots and soil of trees with crown rot in the 30-year-old orchard in Merced County, and a *Phytophthora* sp. was detected from the soil around a declining walnut tree on Paradox in Kings County, but none of the other samples from Paradox yielded a *Phytophthora* sp. or any other known bacterial or fungal pathogen. Many bacteria were isolated by dilution plating from the necrotic bark, but rDNA sequences of subcultured representatives from these isolates were linked only to saprophytic (presumably non-pathogenic) bacteria (Tables 2,3). When necrotic bark samples were ground up and used to inoculate excised walnut shoots, no disease resulted (data not shown).

PCR amplifications of DNA extracted from the necrotic Paradox samples described above also failed to associate the disease symptoms with a pathogen. The primer sets employed either did not amplify DNA from the diseased or healthy control samples or they amplified from both diseased and healthy samples (Table 1). When 95 amplicons (39 from healthy Paradox tissue, 56 from diseased Paradox tissue) from PCR with bacterial primers 63F and 1401R were sequenced, BLAST searches linked the amplicons to host plastids, regardless of whether the DNA was from healthy or diseased bark; apparently the primers were amplifying host DNA.

Several approaches will be taken to gain more insight to the causes of the crown rot on Paradox. It became apparent in the process of sampling that cankers on Paradox in Kings County were active in mid-summer, but not active in May or October. We will therefore intensify orchard surveys in mid-summer in 2009 to improve chances of detecting a pathogen. We will use additional isolation media and methods, and examine the possibility that glyphosate or paraquat herbicides are contributing to the cankers in Kings County. In addition, we recently began using suppression subtractive hybridization (SSH) a PCR-based method that can be used to "subtract out" all DNA except that unique to a phenomenon of interest, in this case, the canker disease on Paradox. The SSH approach should help solve the problem of non-selective amplification of walnut DNA, thereby permitting us to "sort through the haystack" of host DNA and focus on potential pathogen DNA.

Decline associated with other symptoms. The declining trees on Paradox rootstock sampled in Butte County, which had severe root rot but lacked distinct crown cankers, appeared to be suffering from waterlogged soil and physiologically induced root damage rather than attack of an aggressive pathogen. No *Phytophthora* was detected and there was no evidence of Armillaria or crown gall.

Similarly, walnut trees on Paradox rootstock that exhibited some decay of the fine roots but no severe root rot or crown rot in San Joaquin County did not appear to be suffering from invasion of an aggressive pathogen; a *Phytophthora* sp. was isolated from the soil, but not from the roots. It was not clear that *Phytophthora* was associated with the disease.

In contrast to the results above, *Phytophthora* sp., identified by rDNA sequencing to be *P. citricola*, was associated with decline of walnut trees on NCB rootstock in Kings County. Flood irrigation resulting in long cycles of soil saturation with water and the relative susceptibility of the rootstock appeared to be contributing to infection by the pathogen and death of the trees.

Table 1. PCR primers tested for amplification of specific DNA fragments from target organisms

Target microbe	Primer pairs	Annealing Temp. (^{0}C)	DNA amplification	
			Healthy samples	Diseased samples
Fungi	ITS1 & ITS4	60	Yes	Yes
	ITS5 & ITS4	55	Yes	Yes
	ITS1F & ITS4	53	Yes	Yes
Ascomycetes	ITS5 & ITS4Asco	55	No	No
	ITS1F & ITS4Asco	53	No	No
Basidiomycetes	ITS5 & ITS4Basidio	58	Yes	Yes
	ITS1F & ITS4Basidio	55	No	No
Chytridiomycetes	ITS5 & ITS4Chytri	53	Yes	Yes
	ITS1F & ITS4Chytri	53	No	No
Oomycetes	ITS5 & ITS4Oo	49	No	No
	ITS1F & ITS4Oo	49	No	No
Zygomycetes	ITS5 & ITS4Zygo	45	Yes	Yes
	ITS1F & ITS4Zygo	45	Yes	Yes
Bacteria	63F & 1401R	60	Yes	Yes
Agrobacterium	VCF and VCR	49	No	No
Brennaria rubrifaciens	GSP1F & GSP1R	58	No	No
Clavibacter	R16FO and CBR16R1	60	No	No
Erwinia	gap1F & gap1R	60	No	No
Pseudomonas	PA-GS-F & PA-GS-R	54	No	No
Streptomyces	Nf and Nr	60	Yes	Yes
Xanthomonas	16Suni1330 & 23Suni322a	65	Yes	No

Table 2. Identity of culture-isolated bacteria associated with diseased and healthy portions of bark from Paradox rootstock affected by basal trunk and root crown cankers[a] – Sample Set 1

Sample	Trt	Total	Bacterial genus and number of isolates				
			Bacillus	*Burkholderia*	*Paenibacillus*	*Leuconostoc*	Others
gb5833_ Diseased	Bleach	10	8	1	1	0	0
gb5833_ Diseased	Rinse	4	2	0	0	0	2
gb5834_ Diseased	Bleach	16	5	8	1	0	2
gb5834_ Diseased	Rinse	11	2	9	0	0	0
gb5836_ Diseased	Bleach	5	0	5	0	0	0
gb5836_ Diseased	Rinse	5	2	0	0	3	0
Total	-	51	19	23	2	3	4

[a] Bacterial 16S universal primers, 63F and 1401R, were used to amplify a fragment of 1340 bp from DNA of each isolate, and the purified PCR product was sequenced.

Table 3. Identity of culture-isolated bacteria associated with diseased and healthy portions of bark from Paradox rootstock affected by basal trunk and root crown cankers[a] – Sample Set 2

Sample	Trt	Total	Bacterial genus and number of isolates			
			B. megaterium	*B. subtilis*	*B. simplex*	*Bacillus* sp.
gb5869_ Healthy	Bleach & Rinse	15	4	5	0	6
gb5870_ Edge	Bleach & Rinse	14	0	11	0	3
gb5870_ Away	Bleach & Rinse	13	3	7	1	2
gb5871_ Healthy	Bleach & Rinse	14	4	9	0	1
gb5872_ Edge	Bleach & Rinse	15	1	11	0	3
gb5872_ Away	Bleach & Rinse	16	2	9	1	4
Total	-	87	14	52	2	19

[a] Bacterial 16S universal primers, 63F and 1401R, were used to amplify a fragment of 1340 bp from DNA of each isolate, and the purified PCR product was sequenced.

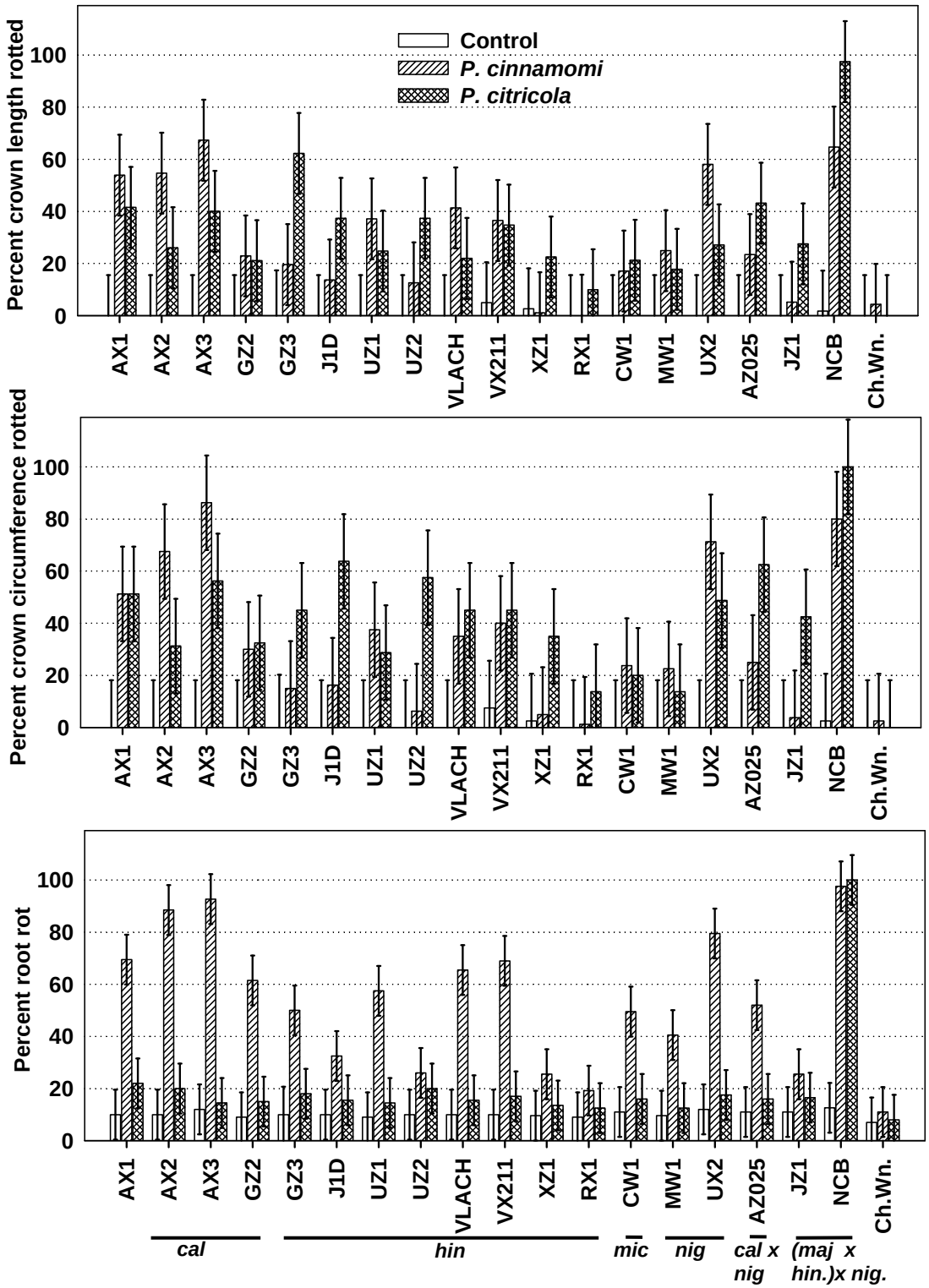

Fig. 1. Relative resistance to *Phytophthora cinnamomi* and *P. citricola* among 17 clonal Juglans hybrid rootstocks, Northern California black walnut (NCB), and Chinese wingnut (Ch.Wn.) in a greenhouse experiment established May 2008. The maternal species background is indicated for each hybrid: *cal=californica*, *hin= hindsii*, *mic= microcarpa*, *cal x nig = californica x nigra*, *(maj x hin) x nig = (major x hindsii) x nigra*. All hybrids had *J. regia* as paternal parent. Vertical bars are 95% confidence intervals.

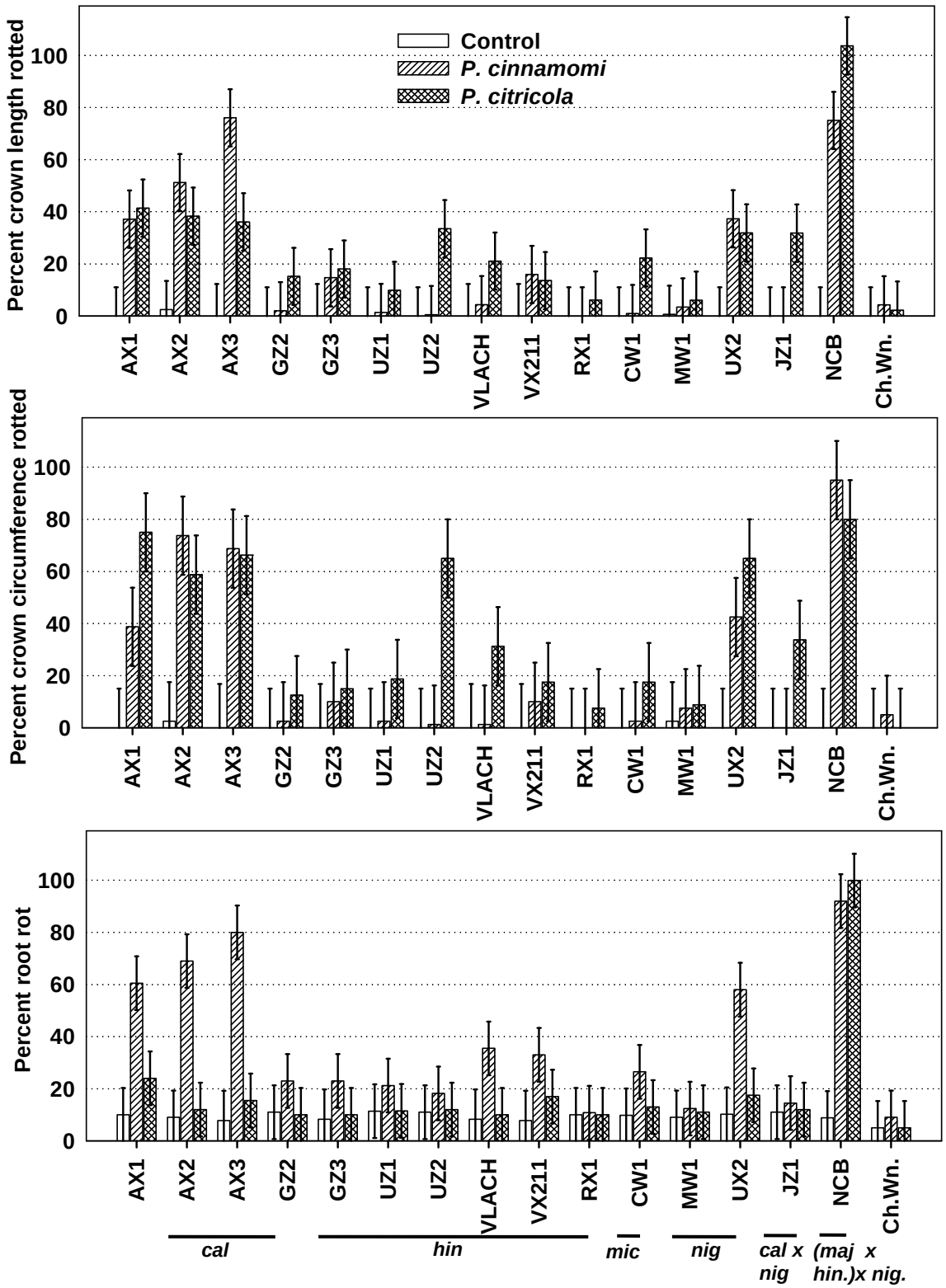

Fig. 2. Relative resistance to *Phytophthora cinnamomi* and *P. citricola* among 17 clonal *Juglans* hybrid rootstocks, Northern California black walnut (NCB), and Chinese wingnut (Ch.Wn.) in a greenhouse experiment established July 2008. The maternal species background is indicated for each hybrid: *cal=californica*, *hin= hindsii*, *mic= microcarpa*, *cal x nig = californica x nigra*, *(maj x hin) x nig = (major x hindsii) x nigra*. All hybrids had *J. regia* as paternal parent. Vertical bars are 95% confidence intervals.