Workgroup/Department Walnut/Plant Pathology

University of California
Division of Agricultural Sciences
Project Plan/Research Grant Proposal

Project Year 2008/09 (May 1, 2009-April 30, 2010) **Anticipated Duration of Project** Yr 2 of 2

Project Leader G.T. Browne **Location** USDA-ARS, Dept. of Plant Pathology

Cooperating Personnel Leigh Schmidt, Ravi Bhat, Nat Quesenberry, D. Doll, Dan Kluepfel, Wes Hackett, Bob Beede, Gale McGranahan, Charles Leslie, Bruce Lampinen, Bill Krueger, and Joe Connell

Project Title Biology and management of Phytophthora crown and root rot on walnut

Keywords Walnut, *Phytophthora*, crown rot, root rot, genetic resistance, rootstock, Paradox,

Commodity Walnut **Relevant AES/CE Project No**.

Problem and its Significance:

The problems addressed by this proposal are: 1) Phytophthora root and crown rot caused by *Phytophthora cinnamomi* and *P. citricola* and 2) a potentially new crown rot disease affecting Paradox rootstock. Background on each of these problems is presented below.

The first problem, Phytophthora crown and root rot, results in serious economic loss in walnuts worldwide, and use of rootstocks with resistance to the causal pathogens is probably the most economical and effective approach for managing the disease. In California, more than 10 species of *Phytophthora* are known pathogens of walnut, but *P. cinnamomi* and *P. citricola* are particularly aggressive and difficult to control (Mircetich et al., 1998). Northern California black walnut (NCB) (*Juglans hindsii*) and English walnut seedling rootstocks (*J. regia*) are highly susceptible to most of the causal species. Paradox seedling rootstocks (typically *J. hindsii* x *J. regia*) are more tolerant than NCB or English rootstocks to Phytophthora but still are moderately to highly susceptible to *P. cinnamomi* and *P. citricola*. *Phytophthora cinnamomi* typically initiates attack through the roots and then advances to the root crown, whereas *P. citricola* invades at the crown of the root system or at the base of the tree trunk. Both pathogens can kill trees within a year or two of infection. To date, the most notable achievement of this project in regards to management of *Phytophthora* is probably the identification of the rootstock clone RX1 (*J. microcarpa* × *regia*), which consistently has expressed moderate to high resistance to both *Phytophthora citricola* and *P. cinnamomi*.

Evidence for the second problem, a potentially new root crown rot disease affecting trees on Paradox rootstock, includes its occurrence in the apparent absence of known causes of crown rot, including *Phytophthora* spp., *Armillaria mellea*, and secondary decay resulting from waterlogging. Compared to NCB, Paradox rootstock typically offers superior tree vigor, resistance to *Phytophthora* spp., and tolerance to parasitic nematodes, but in several orchards, the latter rootstock has suffered decline and death due to root system failure. Although our field surveys have associated some cases of decline on Paradox with waterlogging, *Phytophthora*, or *Armillaria*, others have involved distinct cankers not associated with known pathogens (Browne et al., 2006 and 2007 reports to WMB) or waterlogging. The latter cases have 1) occurred at sites not affected by high water tables or flooding, and 2) have exhibited “spotty” incidence (i.e.,

affected trees are scattered among healthy trees). It is important to determine the cause(s) of the potentially new crown rot disease on Paradox, because it appears to be associated with the death of English walnut trees in many orchards. Although incidence of the disease is low (<1% in affected orchards), it is possible that it will become more prevalent as use of Paradox rootstock continues.

Objectives

1. Evaluate elite hybrid clones for resistance to *Phytophthora cinnamomi and P. citricola*.
2. Examine potentially new causes of crown rot on Paradox rootstock.

Work under Objective 1, detailed under Plans and Procedures (below), will focus on completing greenhouse evaluations of hybrid rootstock clones originating from the Paradox Diversity Study (PDS). The clones evaluated will include new selections that have not been tested previously and items that were selected years ago but have not been thoroughly tested with *P. citricola* or *P. cinnamomi*. Additionally under Objective 1 we will support efforts of D. Doll or another farm advisor in establishing a field trial to evaluate performance of RX1, VX211, and AX1 in an orchard "naturally" infested with *P. cinnamomi*. It is important to note that the evaluations proposed under Objective 1 will be used to justify and guide an additional interdisciplinary team effort: multiple-year funding will be sought in 2009 through the USDA-CSREES Specialty Crop Research Initiative (SCRI) to broaden and intensify research on walnut rootstock genetics and resistance to soilborne pathogens, including *Phytophthora* species. With the additional SCRI funding, this project will work in concert in the interdisciplinary team to evaluate more species and hybrids for their resistance to *Phytophthora* and additional pathogens. Although the SCRI work will examine resistance of diverse walnut family members to *P. citricola* and *P. cinnamomi*, it will emphasize in-depth examination of genetic backgrounds that have yielded superior resistance to pathogens in the PDS. For example, for intensified work within the promising genetic background of RX1, M. Aradhya has harvested maternal and putatively hybrid seed from several sources of *J. microcarpa* for us to test. Additional backgrounds being propagated by Aradhya as seedlings for our testing include *J. ailantifolia* (has yielded resistance to *Phytophthora* spp. previously), *J. cathayensis* (has yielded resistance to lesion nematode), *J. major*, *J. hindsii* (Arizona and Northern CA black walnut, respectively), and *Pterocarya stenoptera* (Chinese wingnut).

Work under Objective 2 will concentrate in Kings County in orchards where the Paradox crown rot has been observed previously with farm advisor B. Beede. As detailed below, samples from the affected trees will be subjected to: 1) bacterial, fungal, and oomycete isolations in culture media and 2) culture-independent polymerase chain reaction (PCR) assays to detect pathogens without culturing. These assays will test the hypothesis that a pathogen is inciting and will be associated with incidence of the crown rot disease. Suspected pathogens will be tested for pathogenicity. In addition, if circumstantial evidence warrants, we will conduct tests with herbicides (i.e., glyphosate, paraquat) to determine if herbicides may incite the crown decay.

Plans and Procedures

Objective 1

Greenhouse trials. We will evaluate resistance of approximately 20 hybrid clones to *P. cinnamomi* and *P. citricola* in greenhouse trials based at UC Davis (Tables 1,2). Some of these

clones have been tested previously for their response to the pathogens, but they will either be tested again to serve as standards for comparison or tested again to confirm previous, tentative responses. Others of the clones have not been tested previously. Each of the evaluations will include AX1 and PX1 (clonal hybrids of *J. californica* × *J. regia* and *J. hindsii* × *J. regia*, respectively) and NCB as standards that are moderately to highly susceptible to *P. citricola*, RX1 (clonal hybrid of *J. microcarpa* × *J. regia*) as a standard moderately resistant to *P. citricola*, and Chinese wingnut seedling (*Pterocarya stenoptera*) as a standard highly resistant to both *P. cinnamomi* and *P. citricola*.

The experimental Paradox selections will be prepared for greenhouse evaluations of resistance by the WIP under supervision by Hackett and Browne as indicated in the 2009/10 WMB proposal "*Clonal propagation of walnut rootstock genotypes for genetic improvement*" from McGranahan et al. The selections will be cycled through dormancy induced by refrigerated storage (several months at 42 °F) or by overwintering in a lath house, depending on the experiment. A cycle of dormancy tends to equalize the physiological status of the diverse clones and increases "physiological susceptibility" to the pathogen after the plants resume growth. After the chilling, the plants will be moved to a greenhouse and watered and fertilized as needed as they break dormancy and establish vigorous shoot and root growth.

Two to 3 months after emergence from dormancy, under the supervision of Browne, the plants will be transplanted into 2-liter pots of non-infested UC potting soil mix (UCM) and UCM artificially infested with the appropriate *Phytophthora* sp. (40 ml of colonized V8 juice-vermiculite-oat medium per liter of soil). The experiments will employ a split-plot design with the inoculum treatments randomly allocated to main plots (i.e., groups of individual potted plants that include each hybrid clone and standard) in complete blocks and the rootstock plants randomly allocated to subplots (individual pots, each to hold a plant). In each experiment, 5 to 20 plants per rootstock will be inoculated with *P. cinnamomi*, another 5 to 20 with *P. citricola*, and 5 will serve as noninoculated controls. Starting 1 week after transplanting, the plants will be subjected to 48-hr episodes of soil flooding, once every 2 weeks. Between flooding episodes, plants will be watered and fertilized to optimize growth. Plants will be rated for severity of aboveground disease symptoms every 2 weeks using a 0-to-5 scale (i.e., 0=healthy, 5=dead, 1,2,3, and 4=progressive increments of disease). Disease progress curves will be calculated using the biweekly disease severity ratings. About 3 months after transplanting, the plants will be washed free from soil and evaluated for severity of crown and root rot. The proportion of the root crown length and circumference rotted will be measured on each plant and expressed as percentages. The percentage of roots rotted will be visually estimated. Root and shoot fresh weights will be determined.

All data will be subjected to analysis of variance and appropriate mean separation procedures. Clones exhibiting relatively little crown and root rot in repeated greenhouse trials will be considered tolerant to the pathogens and, barring unacceptable vulnerabilities or attributes, worthy of intensive field trials and orchard evaluations.

Orchard trial. We will work with D. Doll and other UCCE advisors to locate an orchard site infested with P. cinnamomi for a rootstock trial to be planted in Jan. 2010. Isolations and/or results of previous isolations will be used to confirm infestation status of the site. We will advise and collaborate with Doll to design an appropriate trial with approximately 6 replicate blocks including rootstocks RX1, AX1 or PX1 and VX211.

Objective 2.

We will concentrate examination of the Paradox crown rot in Kings County where it has been observed previously in several orchards. Samples of necrotic crown and root tissue will be collected and cultured in semi-selective and non-selective media to detect oomycetes (e.g., *Phytophthora* and *Pythium* spp.), fungi, and bacterial pathogens. Potentially pathogenic isolates of *Phytophthora* and fungi associated with the disease will be tested for pathogenicity on Paradox hybrids and NCB in the greenhouse using methods similar to those of greenhouse screens for resistance. Isolates of bacteria will be tested for pathogenicity by inoculating suspensions of them from pure tryptic soy broth agar cultures onto wounds on stems of Paradox and NCB rootstock seedlings. A cork borer will be used to remove a small patch of bark from greenhouse and field plantings of the plants, and a quantified volume of bacterial suspension will be placed on the wounds and wrapped to prevent drying. The wounds will be allowed to incubate for approximately 4 weeks and then examined for evidence of necrosis. Controls will be inoculated with sterile broth. Isolations and identifications will be conducted from the necrotic tissues to complete Koch's postulates. Dan Kluepfel will collaborate with us in characterizing the bacterial isolates and their pathogenicity.

Culture-independent (i.e., PCR-based) assays also will be completed for the samples described above. We will use suppression subtractive hybridization (SSH) (Lukyanov et al., 2007) to selectively amplify DNA associated with the diseased condition of necrotic crown tissue sampled from Paradox rootstock. Briefly, samples will be collected from two areas on trees on Paradox rootstock affected by crown rot: 1) healthy Paradox bark at least 20 inches distant from the crown rot and 2) diseased Paradox bark just inside the necrotic margin of the crown rot. Genomic (i.e., total) DNA will be extracted and purified separately from each of the two samples. Then, using the SSH protocol, which involves DNA restriction, ligation with adapters, hybridization steps, and selective PCR, DNA from the healthy sample will be used to hybridize with and "subtract" DNA from the diseased samples that has sequences in common, theoretically leaving only DNA associated with the diseased condition available for PCR amplification. The latter DNA fragments can be cloned and sequenced to identify their microbe sources. The SSH protocol also will be focused on diagnostic regions of choice in genomic DNA samples (i.e., rDNA or other regions well represented by sequences on the National Center for Biotechnology Information sequence database) to aid in identification of pathogens' DNA that may be present in the samples.

As field observations and results justify, we also will pursue alternative hypotheses on the cause of the crown rot on Paradox. Bark and cambium samples from the canker margins will be collected in buffer solutions and used for herbaceous host assays for the presence of virus. Also, according to herbicide use circumstances in the affected orchards, suspect herbicides such as glyphosate and paraquat will be tested with appropriate controls to determine if herbicide damage may induce the crown rot. Workers in the northeastern US have suspected that crown rot on apple rootstocks resulted from exposure to glyphosate and/or paraquat formulations, however, the symptoms on apple do not closely resemble those on walnut (Rosenberger and Fargione, 2004).

Collaboration note: In addition to the work proposed here, Browne is eager to collaborate in *"Evaluation of elite hybrid clonal rootstocks for resistance to Phytophthora root and crown rot: Mechanisms of root stress predisposition to disease"* in the related project proposed by Bostock.

Literature Cited

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Rosenberger, D., Fargione, M. 2004. Apple tree deaths attributable to herbicides? In: Diseases section of Scaffolds Fruit Journal Vol. 13 No. 13; http://www.nysaes.cornell.edu/ent/scafolds/2004/040615.html#disease

Budget Request

Budget Period: May 1, 2009 to April 30, 2010
Funding Source: Walnut Marketing Board

Item	**Amount ($)**
Post graduate researcher / SRA salary and benefits:	16,000
Supplies and Expenses:	2,000
Travel:	500
Total:	**18,500**

Signature of Principal Investigator: _____

Signature of Authorized Institution Rep,
USDA-ARS, Davis; Location Coordinator: _____

Signature of Authorized Institution Rep._____

University of California, Davis; Dept. Chair:_____

Table 1. Completed and proposed screening with *Phytophthora citricola* in 2009/10[a]

Genetic background (all *Juglans* specified otherwise)			Experiment and resistance to *Phytophthora citricola* as measured by severity of crown rot								Proposed for screening w/ *P. citricola* in 2009/10
Maternal	**Paternal**	**Clone**	**2001**	**2004a**	**2004b**	**2006**	**2007a**	**2007b**	**2008a**	**2008b**	
ailantifolia	*regia*	DAR									+
(major x hindsii) x nigra	*regia*	NZ1		MR	MR	MR	MS				
(major x hindsii) x nigra	*regia*	AZ025					MS	MS	MR		
(major x hindsii) x nigra	*regia*	AZ1	MR			MR	MS				
(major x hindsii) x nigra	*regia*	AZ2	MR	MR	MR	MR	MS				
(major x hindsii) x nigra	*regia*	AZ3		MR	MR	MR	MS				
californica	*regia*	AX1	MS	MS	MS	MR	MR	MS	MR	MR	+
californica	*regia*	AX2	MS				MS	MS	MR	MR	+
californica	*regia*	AX3							MR	MR	+
californica x nigra	*regia*	UX022				MS	MS				
californica x nigra	*regia*	UX1	HR			MR	MS	MR			+
californica x nigra	*regia*	UX2	MS			MS	MS	MR	MR	MR	+
hindsii	*hindsii*	BUR					MR	MS			
hindsii	*regia*	GZ1		MS	MS	MS	MS				
hindsii	*regia*	GZ2				MR	MS	MS	MR	MR	
hindsii	*regia*	GZ3					MS	MS	MS	MR	+
hindsii	*regia*	JX1							MR	MR	
hindsii	*regia*	JX2	MR	MR	MR	MR	MS				
hindsii	*regia*	PX1		MS	MS	MR	MS	MS			+
hindsii	*regia*	RR1					HS	MS			
hindsii	*regia*	RR4					HS	MS			+
hindsii	*regia*	UZ1							MR	HR	+
hindsii	*regia*	UZ2							MR	MR	+
hindsii	*regia*	84-121	MR								
hindsii	*regia*	Vlach				MR	MS	MS	MR	MR	
hindsii	*regia*	VX211		MR	MR	MR	MS	MR	MR	MR	+
hindsii	*regia*	J1D							MR		+
hindsii	*regia*	XZ1							MR		
hindsii x regia	*regia*	WIP2				MS	MS				+
hindsii x regia	*regia*	WIP3		MS	MS	MS	HS	MS			+
hindsii x regia	*regia*	WIP4									+
hindsii x regia	*regia*	WIP6									+
microcarpa	*regia*	RX1	HR	MR	MR	(MS)	MR	MR	HR	HR	
nigra	*regia*	CW1					MS	MS	MR	MR	
nigra	*regia*	MW1					MS	MS	MR	HR	+
nigra	*regia*	RW2					MS	MS			
nigra	*regia*	CR					MS	MS			
hindsii	*hindsii*	--				HS	HS	HS	HS	HS	+
Pterocarya stenoptera	*P. sten.*	--					HR	HR	HR	HR	+

[a]Abbreviations: MS=moderately susceptible, with mean of 51 to 90% crown length rotted; MR=moderately resistant, with mean of 11 to 50% crown length rotted; HR=highly resistant, with mean of 0 to 10% crown length rotted; and HS= susceptible with mean of 90 to 100% of crown length rotted.

Table 2. Completed and proposed screening with *Phytophthora cinnamomi* in 2009/10[a]

Genetic background (all Juglans unless otherwise specified)			Experiment and resistance to *Phytophthora cinnamomi* as measured by severity of root rot			Proposed for screening w/ *P. cinnamomi* in 2009/10
Maternal	**Paternal**	**Clone**	**2007**	**2008 a**	**2008 b**	
ailantifolia	*regia*	DAR				+
(major x hindsii) x nigra	*regia*	NZ1				
(major x hindsii) x nigra	*regia*	AZ025	MR	MS		
(major x hindsii) x nigra	*regia*	AZ1				
(major x hindsii) x nigra	*regia*	AZ2				
(major x hindsii) x nigra	*regia*	AZ3				
californica	*regia*	AX1	MS	MS	MS	+
californica	*regia*	AX2	HS	HS	MS	+
californica	*regia*	AX3		HS	MS/HS	+
californica x nigra	*regia*	UX022				
californica x nigra	*regia*	UX1	MR			+
californica x nigra	*regia*	UX2	HS	MS	MS	+
hindsii	*hindsii*	BUR	MR			
hindsii	*regia*	GZ1				
hindsii	*regia*	GZ2	MR	MS	MR	
hindsii	*regia*	GZ3	MS	MS/MR	MR	+
hindsii	*regia*	JX1				
hindsii	*regia*	JX2				
hindsii	*regia*	PX1	MS			+
hindsii	*regia*	RR1	MR			
hindsii	*regia*	RR4	MS			+
hindsii	*regia*	UZ1		MS	MR	+
hindsii	*regia*	UZ2		MR	MR	+
hindsii	*regia*	84-121				
hindsii	*regia*	Vlach	MS	MS	MR	
hindsii	*regia*	VX211	MR	MS	MR	+
hindsii	*regia*	J1D				
hindsii	*regia*	XZ1				
hindsii x regia	*regia*	WIP2				+
hindsii x regia	*regia*	WIP3	MS			+
hindsii x regia	*regia*	WIP4				+
hindsii x regia	*regia*	WIP6				+
microcarpa	*regia*	RX1	HR	MR	HR	+
nigra	*regia*	CW1	MS	MR	MR	
nigra	*regia*	MW1	MR	MR	HR	+
nigra	*regia*	RW2	HS			
nigra	*regia*	CR	HS			
Hindsii	*Hindsii*	--	HS	HS	HS	+
P. Stenoptera	*P. stenoptera*	--	HR	HR	HR	+

[a]Abbreviations: MS=moderately susceptible, 51 to 80% mean root rot; MR=moderately resistant, with 15 to 50% mean root rot; HR=resistant, with 0 to 15% mean root ; HS= highly susceptible with 91 to 100% mean root rot.