University of California
Division of Agricultural Sciences

PROJECT PLAN/RESEARCH GRANT PROPOSAL

Project Year **2009** Anticipated Duration of Project **2 years**

Project Leader **Richard M. Bostock** Location **Dept. of Plant Pathology/UC Davis**

Cooperating Personnel **Greg Browne, USDA-ARS/Plant Pathology/UCD; Wes Hackett, UCD Plant Sciences; Tatiana Roubtsova, UCD Plant Path**

Project Title **Evaluation of elite hybrid clonal rootstocks for resistance to *Phytophthora* root and crown rot: Mechanisms of root stress predisposition to disease**

Keywords **abiotic stress; abscisic acid; ethylene; germplasm; waterlogging;**

Commodity(s) **Walnut** Relevant AES/CE Project No.

Problem and its Significance:

Phytophthora root and crown rot (RCR) of walnuts is a long-standing and serious problem for California walnut growers. Research targeting the development of resistant rootstocks and effective disease management strategies are high priorities for the industry. UC and USDA-ARS plant pathologists over the course of many years of research have established causal species involved and the environmental factors that influence disease development. Recent efforts are focused on the development of improved rootstocks with resistance to *Phytophthora* RCR, as well as other important soilborne diseases such as crown gall, *Armillaria* root rot, and lesion nematode. It is generally agreed that the most effective and sustainable approach to managing these diseases in walnut will be through genetic resistance.

The interaction of waterlogging in the development of *Phytophthora* RCR in walnut is a classic example of disease predisposition. Predisposition is **"**The tendency of non-genetic factors, acting prior to infection, to affect the susceptibility of plants to disease."(Yarwood 1959) Abiotic stress strongly influences plant resistance to pathogens and, depending on the host-pathogen interaction and stress intensity, the stress can induce susceptibility to pathogens the plant would otherwise resist (Schoeneweiss 1981; Boyer 1995). Classic studies of predisposition in root and crown rot diseases caused by *Phytophthora spp.* demonstrated that cycles of soil saturation (low O_2), drought, and salinity markedly increase disease severity (Duniway 1977; Kuan and Erwin 1980; Macdonald 1982; Macdonald 1984; Browne and Mircetich 1988; Ristaino and Duniway 1989). This research defined many of the physiological parameters of stress-induced predisposition in laboratory, greenhouse and field experiments and illustrated that significant predisposition can occur from relatively minor stresses (e.g., brief exposure to water potential deficits of -0.5 to -1 MPa). This research also has shown that the *Phytophthora* spp. of concern function effectively in soil during (or, in the case of drought, immediately after) the stresses that cause predisposition (Blaker and Macdonald 1985; Ristaino and Duniway 1989). Importantly, the stress levels that are thresholds for predisposition occur routinely in agricultural and non-agricultural systems**,** and, in the absence of a pathogen, plants usually recover rapidly and fully when the stress is relieved (Boyer 1995). A practical outcome of this research was recognition of the importance of predisposition to realistically assess plant performance against root pathogens. This is exemplified by the incorporation of "stress-screens" in some commercial plant breeding programs as

well as in the current protocol for screening walnut genotypes for resistance to RCR where flooding episodes are used (Heritage and Harrigan 1984; Browne et al. 2007).

Although multiple species of *Phytophthora* can cause RCR in walnut, two species, *P. cinnamomi* and *P. citricola*, are most aggressive and are being used by the Browne lab (USDA-ARS/UC Davis) in their rootstock-screening program. Dr. Browne and colleagues are examining the significant genetic diversity among Paradox hybrids as a potential source of resistance against these *Phytophthora* species. They are working with the Walnut Improvement Program, commercial nurseries and Prof. Wes Hackett (Plant Sciences, UC Davis) to systematically screen clonal selections. The hybrids, AX1, PX1, and RX1, have served as standards for comparison in the evaluations. Northern California black (NCB; *J. hindsii* X) rootstocks are highly susceptible to RCR, and Chinese Wingnut (*Pterocarya stenoptera*) is highly resistant and presents a potential source of strong genetic resistance for introgression. These and other clones identified in the screening program provide a range of phenotypes to RCR that are useful for comparison with respect to the objectives of this proposal.

Research on root stress predisposition to *Phytophthora* with other plant species is beginning to reveal important features of the regulatory network in the plant that modulates the severity of infection. Predisposing abiotic stresses are clearly channeling their effect on disease resistance through phytohormone pathways, notably abscisic acid (ABA) and possibly ethylene (Bostock 2005; Asselbergh et al. 2008; Yasuda et al. 2008). Research on *Phytophthora* root rot in my lab is seeking to identify critical features of this "predisposition" network, with the intent to identify targets to improve disease resistance under root stress. Studies with tomato, *Rhododendron*, and other species have shown that anoxia, salinity, and drought impose a degree a water stress in the plant and all have the potential to induce ABA (Zeevaart and Creelman 1988; Munns 2002). Although responses to abiotic stress are complex, we are testing genes that interfere with programmed cell death (Wang et al. 1996; Dickman et al. 2001; Lincoln et al. 2002; Li and Dickman 2004) and ABA response to see if they protect against disease following episodic root stress. Most recently our studies have used salinity stress because of the ease of imposing the stress in a synchronous and uniform manner. Our key findings that implicate ABA in predisposition to *Phytophthora* root rot include:

- Plants are predisposed by brief exposure of the roots to salt stress (0.1-0.2 M NaCl plus $CaCl_2$; equivalent to -0.5 to -1.0 MPa). The effect is similar whether roots are exposed to the inoculum prior to or immediately after the stress episode. Non-inoculated but stressed plants recover completely.
- Exogenous ABA can substitute for the NaCl treatment, and ABA increases rapidly in tomato roots following exposure to NaCl (Bostock et al. 1990).
- Roots of ABA-deficient tomato mutants accumulate only a small fraction of the ABA present in wild-type roots, and they do not show the adaptive responses to water stress (Taylor and Tarr 1984). Since the mutants do not accumulate high levels of ABA in response to root stress, under equivalent stresses they are less "predisposable" to disease than the wild type (Pye et al. 2008).
- We have studied ABA and salinity stress in various interactions with similar results, including tomato vs. a foliar bacterial pathogen, several insect herbivores (Thaler and Bostock 2004), and the root-knot nematode, *Meloidogyne javanica* (Roubtsova and Bostock, unpublished), and with *Phytophthora ramorum* in woody ornamentals (Roubtsova and Bostock 2008).

Walnut roots likely respond to predisposing root stress in a manner similar to other species, but the extent to which this is so and whether there are genotypic differences that correlate with differences in RCR resistance is unknown. Research by UCD plant scientists many years ago examined the effects of waterlogging on root physiology of walnut species and hybrids, including NCB, Paradox, as well as the related Chinese wingnut (Catlin and Olsson 1986; Walnut research reports 1973-81; http://walnutresearch.ucdavis.edu/). Walnut shoot ABA levels increase dramatically within the first 12-18 h of waterlogging in *J. nigra* and decline by 30 h, a classic ABA response to root stress (Shaybany and Martin 1977). To my knowledge, this aspect has never been examined further, and certainly not from the standpoint of how altered ABA levels may compromise disease resistance in this species. A comparative study examining the responses of clonal rootstocks showing a range of RCR resistance and

susceptibility following waterlogging would be informative. A rapid assay for assessing root responses also would help move the work forward.

We have developed high-throughput predisposition seedling assays in other species. For example, in tomato, stress treatment, inoculation and evaluation can be conducted in 72-96 hr. Measurement of colonization by *Phytophthora capsici* is performed by quantitative PCR, which corresponds well with disease severity. We believe that an assay similar to this could be adapted for walnut seedling roots to study predisposition to *Phytophthora* RCR by episodes of anoxic stress, simulating the physiological effect of waterlogging. Such an assay conceivably could be adapted as a complementary approach to help focus the selection of germplasm for screening by traditional methods, which may take weeks or months to complete. Importantly, the discovery work proposed here could position walnut as a tractable system to explore candidate genes now being tested in other systems for impact on stress tolerance and resistance to *Phytophthora* RCR. The resources and collective expertise focused on walnut rootstock improvement at UCD presents a unique opportunity for collaboration and innovation.

Objectives:

1. Develop a small format walnut seedling assay for rapid assessment of *Phytophthora* RCR and physiological responses following anoxic stress.
2. Evaluate a collection of the elite Paradox hybrid clones (moderately resistant to moderately susceptible), NCB (*J. hindsii*; highly susceptible), and Chinese wingnut (highly resistant) to see if the small format assay correlates with results from greenhouse seedling assays.
3. As informed by the outcomes of objectives 1 & 2, evaluate hybrid clones for their responses to anoxic stress, initially focusing on ABA and other known physiological/biochemical responses to anoxic stress and damage.

Plans and Procedures:

Objective 1. Walnut seedling assay development. Most of the work during the first year of this project will focus on development of a small seedling assay for predisposition studies. Initially, we will use a highly susceptible rootstock like NCB for assay development and proof of concept. The Hackett lab at UCD can routinely produce aseptically-grown rooted and unrooted clonal rootstock microshoots in vermiculite. We will pursue this format first. Another option could be the use of excised walnut embryos in vermiculate for aseptic seedlings, if contamination proves to be an issue. The basic disease assay will be an abbreviated version of that used in the traditional walnut screens by the Browne lab, or with variation as appropriate. The plants will be transferred to cups containing vermiculite artificially infested with a V8 juice-oat-vermiculite substrate infested with *P. citricola* or *P. cinnamomi* in the proportions used in the traditional disease assay. Controls will be treated with sterile substrate. Roots then will be submerged for 48 h, the time typically used for predisposition to RCR in walnut, recognizing that we may need to test various flooding periods to achieve predisposition. Controls will include inoculated and noninoculated plants that are not exposed to flooding. The plants will be maintained in a growth chamber under appropriate lighting and temperature regimes, and monitored for symptom development. With the small format assay, an alternative inoculation method that might be feasible is the use of zoospore inoculum. Zoospores can be counted, thus providing a known inoculum density for inoculation. The small format assays with rooted microshoots could be designed so as to permit the use of inoculum levels in a sufficient volume that can be obtained feasibly and provide optimal contact with the roots. In all cases substrate and treatment volumes will be carefully controlled to maintain uniformity.

If necessary there are other formats that could be explored as well. For example, excised roots have been used with other species to study the impact of anoxic stress on root physiology, the advantage being that environmental conditions and treatments can be well-controlled and imposed in a uniform and synchronous manner (Goggin and Colmer 2005). Similarly, root systems removed from small walnut plants will be immersed in an appropriate solution (e.g., 0.5x Hoaglands solution). I anticipate that the excised roots could be suitable for use for several days to perhaps a week. If bacterial contamination

poses an issue, an antibiotic such as ampicillin, which does not interfere with *Phytophthora* infection and growth, can be added to the treatment solutions. To simulate the effect of waterlogging, hypoxic root treatment solutions will be generated by bubbling nitrogen to purge the air. Oxygen levels of the medium will be monitored with an O_2 electrode. Excised roots will be exposed to the hypoxic solution for various times, the solution replaced with aerated medium, and inoculated with zoospores with the appropriate controls as describe above.

We will work closely with the Hackett and Browne labs during assay development to help us coordinate experiments and to learn from their expertise. We also will consult other plant stress researchers with expertise on anoxia to ensure that our treatment protocols are sufficiently robust and have the appropriate checks.

Quantitation of disease. Disease development based on symptom expression will be an important rating criterion initially. We will also investigate the use of quantitative PCR (qPCR) for measuring colonization of walnut seedlings by *P. cinnamomi* or *P. citricola* and validate this protocol by comparison with visual ratings. My lab uses qPCR routinely for measuring colonization of plants by *P. capsici* and *P. ramorum*, so I anticipate that this method will be successfully adapted for use in walnut. Using this method, we can measure differences in pathogen DNA content as an indicator of growth and colonization within the host very early in disease development, greatly speeding up the evaluation. Briefly, plant tissues to be analyzed are flash frozen in $N_2(l)$, macerated with a FastPrep tissue homogenizer (MP Biomedicals) and extracted with DNeasy® Plant Mini kits (Qiagen). Pathogen DNA is quantified with a 7500 real-time PCR machine (Applied Biosystems) with established primers (Silvar et al. 2005; Schena et al. 2008). Standard curves will be verified for this system with pure DNA from *P. cinnamomi* or *P. citricola* as appropriate and with *Phytophthora* DNA amended with walnut DNA. In addition, plant actin primers provide an internal control for normalizing results relative to host DNA recovered with each sample.

Objective 2. Evaluate a collection of the elite Paradox hybrid clones and other materials in the small format assay. The utility of the seedling assay developed under objective 1 will be validated by comparing the responses of NCB, hybrid clones, and Chinese wingnut in this assay with previous results using the traditional method from the Browne lab that have shown these to range from highly susceptible to highly resistant to *Phytophthora* RCR.

Objective 3. Assessment of ABA and other known physiological/biochemical responses to anoxic stress and damage.[1] With a rapid, quantitative disease predisposition assay in place, we will assess the physiological/biochemical responses of walnut roots to simulated waterlogging. We will focus on ABA for the reasons explained above, and measure changes in ABA content of roots following predisposing stress. Questions that we can ask include – Do the differences observed in susceptibility to RCR correspond to changes in ABA following anoxic stress? Is the variation in disease resistance in the materials listed under objective 2 related to a greater physiological tolerance to anoxia, or do they differ primarily because of inherent differences in disease resistance regardless of stress? Other root responses such as those described by (Goggin and Colmer 2005) could be examined if time and funds permit.

ABA analyses. ABA concentrations are determined with a procedure we have developed for use in tomato. Root and stem tissues are collected and immediately frozen in liquid N2. The tissues are lypophilized and placed at -20 C until extraction. The lypophilized tissue is ground in liquid N2 to a fine powder with a mortar and pestle, and three 50-100 mg samples are collected and placed in microfuge tubes. ABA is extracted from the powdered samples in cold 80% methanol (1.2 ml) containing 10 µg/ml BHT, and, after a series of steps, the partially purified extracts are applied as a 70% methanolic extract to Sep-pak C18 columns and eluted with 70% methanol. The eluate containing ABA is concentrated to near dryness under vacuum and the volume adjusted to 300 ul with distilled water. The samples are analyzed by a commercial immunoassay using monoclonal antibody specific for (+)- ABA (Agdia Inc.;

[1] We will proceed with objective 3 if the outcomes of the experiments described under the first two objectives justify it.

Cahill and Ward 1989).

Agonists/Antagonists of ABA synthesis/ action. The small format assays will permit evaluating the role of ABA by treating the tissues with agonists (promote) or antagonists (inhibit) of ABA synthesis or action. Exogenous ABA and ABA metabolism inhibitors such as aminotriazole and fluridone will be tested over a concentration range to see if they promote or interfere with root stress predisposition. Although this is a classical approach, these experiments could be informative and corroborate results obtained above. Furthermore, they will provide a framework to justify and guide research into targeted, contemporary approaches to modify rootstock resistance to RCR such as transformation or RNAi. The approaches proposed for ABA also provide a template for examining other candidate molecules that may play a critical role in disease predisposition (e.g., ethylene).

Timetable and work plan: Most of year 1 will be focused on objective 1. We anticipate that the studies under objective 2 will commence late in year 1 and continue into year 2. If warranted based on the outcomes of objectives 1 & 2, the mechanistic studies described in objective 3 will be conducted primarily during year 2 of the project. Assistant Specialist Tatiana Roubtsova will have primary responsibility for conducting this research, with general guidance from me and with assistance from student help. I am asking for partial support (0.3) for her salary and benefits, the remainder being made up from other funding sources I am seeking to support research related to this topic. We will work closely with members of the Browne and Hackett labs as necessary.

Anticipated outcomes. A desirable outcome of this research is that the small seedling predisposition assay corresponds well with the traditional disease assay, and provides a rapid (<1 or 2 wk) assessment of the relative resistance of different walnut rootstock genotypes. I anticipate that the assay will permit tighter control of treatment parameters that are otherwise difficult to maintain under greenhouse conditions and even more so in the field. The assay could provide an initial screen to select the most promising and resistant lines for further testing in the larger plant assay formats. Additional selection criteria may be revealed by the physiological studies. If our studies indicate that susceptibility to *Phytophthora* RCR in walnut is strongly controlled by an ABA-mediated process, then future research could examine how work ongoing in other host-pathogen interactions on this topic can be translated for walnut rootstock improvement.

Note: I will also submit this proposal to the California Improvement Advisory Board for consideration to seek their support and partnership with the Walnut Board.

BUDGET REQUEST Budget Year

Funding Source

Salaries and Benefits

Postdocs/RA's	_____	$_____
SRA's	**T. Roubtsova (Assist. Specialist/0.3 FTE)**	**$ 13,320**
Lab/Field Assistance	_____	$_____

Subtotal	Sub 2	$_____
Employee benefits	Sub 6	$ **6,052**
__	SUBTOTAL	$ **19,372**
Supplies and Expenses	Sub 3	**$ 5,000**
Equipment	Sub 4	$_____
Travel	Sub 5	$_____
	TOTAL	$ **24,372**

Department account number _____

Richard M. Bottorf

_____ Date **12-1-08**

Originator's Signature

COOPERATIVE EXTENSION County Director _____ Date _____

Program Director_____ Date _____

Thomas R. Gordon

AGRICULTURAL EXPERIMENT Department Chair_____ Date **12-1-08**

STATION

LIAISON OFFICER _____ Date _____

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