FINAL PROJECT REPORT
WTFRC Project Number: CP-16-104

Project Title: Phenotyping resistance traits of apple rootstock to replant pathogens

PI:	Yanmin Zhu	**Co-PI:**	Mark Mazzola
Organization:	TFRL-ARS-USDA	**Organization:**	TFRL-ARS-USDA
Telephone:	(509) 664-2280	**Telephone**	(509) 664-2280
Email:	yanmin.zhu@ars.usda.gov	**Email:**	mark.mazzola@ars.usda.gov
Address:	1104 N. Western Ave Wenatchee, WA 98801	**Address:**	1104 N. Western Ave Wenatchee, WA 98801

Cooperators: Gennaro Fazio, apple rootstock breeder, USDA ARS

Agency Name: USDA ARS Tree Fruit Research Lab
Amount awarded: **Year 1: $**55,000 **Year 2: $**55,000

Budget history:

Contract Administrator: Charles Myers, Extramural Agreements Specialist		
Email:cwmyers@pw.ars.usda.gov		
Item	**Year 1**	**Year 2**
Salaries *	38,790	38,790
Benefits	13,577	13,577
Wages		
Benefits		
Equipment		
Supplies	1,733	1,733
Travel		
Miscellaneous		
Total	54,000	55,000

*The **salaries and benefits** are budgeted for a GS-7 technician dedicated to this project.

OBJECTIVES

1. Resistance response evaluation to multiple components of the ARD pathogen complex. Using established protocols for phenotyping the resistance response to *Pythium ultimum* infection, characterization of resistance responses will be expanded to other key components within the ARD pathogen complex including *Rhizoctonia solani* and *Pratylenchus penetrans*.

2. Field performance validation. The greenhouse based phenotype data will be evaluated at a replant site at the Columbia View (CV) experimental orchard. Field performance trials will be carried out using selected genotypes of both susceptible and resistant genotypes based on greenhouse data.

3. Phenotypic data will be used to improve the localization of previously mapped QTLs associated with ARD resistance. The resulting plant materials possessing reproducible and reliable phenotypes will be used in future gene-trait association studies.

SIGNIFICANT FINDINGS

- Individual genotypes from 'Ottawa 3' x 'Robusta 5' (O3R5) progeny were identified with a wide spectrum of resistance levels. More than sixty genotypes were assayed at least twice for response to *P. ultimum* infection. Forty-three genotypes were evaluated for their response to *R. solani* infection and more than thirty genotypes have been assayed for their ability to repel/attract nematodes *P. penetrans*.

- In assessment of the resistance response to *P. ultimum* infection, a primary focus of this work, the survival rates among individual genotypes ranged from 92% to 8% among genotypes. Reductions in root biomass resulting from *P. ultimum* inoculation varied from 5% to 60% and shoot biomass reduction varied from 10% to 60% between the most resistant and the most susceptible genotypes, respectively.

- Contrasting patterns of necrosis progression along the infected roots were associated with the resistant or susceptible phenotype, respectively. Rapid and undeterred necrosis progression was often observed along the roots of susceptible genotypes, and impeded necrosis progression was commonly associated with the roots of resistant genotypes.

- Substantial overlap was observed between resistance to *P. ultimum* and *R. solani* AG-5 but no correlation was found between resistance to *P. ultimum* and *P. penetrans* (nematode).

- Results from preliminary field evaluation at the replant site in Columbia View orchard suggests that the susceptible genotypes benefit more from soil fumigation than resistant genotypes do.

- Current phenotyping dataset of resistance to *P. ultimum* infection was used for detecting resistance QTL in collaborator's lab. However, no major QTLs were identified.

RESULTS AND DISCUSSION

By implementing an in-house plant micropropagation procedure and standardized infection protocol, more than sixty genotypes from O3R5 progeny were repeatedly assayed for their resistance responses towards infection by *P. ultimum.* Forty-three genotypes were also assayed for their response to infection by *R. solani*, and thirty-five genotypes were assayed for their resistance to infestation by *P. penetrans*. For pathogen of *P. ultimum*, the primary focus of this study, overall resistance levels were initially assessed based on overall survival rate of infected plants at 14 dpi (day post inoculation). Plant biomass reductions were determined at 28 dpi by comparison of root or shoot fresh weights between control and infected plants. Root necrosis patterns were examined under dissecting microscope at the early stage of infection, from one to ten dpi. Response to infection by *R. solani*, root infestation by the lesion nematode *P. penetrans*, as well as differential responses to chemical fumigation at a replant site, were also evaluated for selected O3R5 genotypes. The implemented tissue culture procedure enabled the first systematic and detailed analyses on apple root responses to individual components of ARD pathogen complex under controlled experimental conditions. The high-quality resistance phenotypes data of O3R5 population represents an important step for maximized exploitation of host resistance from apple root for managing ARD in future.

1. Wide-range and repeatable resistance responses to *P. ultimum* infection among O3R5 genotypes

Using a standardized phenotyping protocol and repeated infection assays, our data demonstrated a wide-spectrum of plant survival rates due to infection by *P. ultimum* among O3R5 genotypes (**Figure 1**). The top-10 most resistant and susceptible genotypes are listed in **Table 1**. In this study, "resistant" genotypes were assigned to those O3R5 progeny with average survival rate greater than 80%; those progeny with average survival rate lower than 30% were designated as "susceptible. The germplasm genotypes with intermediate values of survival rates were not included in this report.

Figure 1. Distinctive responses among O3R5 progeny to infection by *P. ultimum*. A. Three genotypes showing various responses to *P. ultimum* infection. **B.** Two genotypes, #161 and G.935, exhibiting highly resistant responses. **C.** Two genotypes, #115 and #132, exhibiting more susceptible phenotypes. Plants were inoculated by root dipping method; and inoculum solution contains 2 x 10^3 per mL oospores. All genotypes in Figure 1A were assayed at the same time; all genotypes in B and C were assayed at the same time. Most of these genotypes were assayed for 3-5 times with comparable results. The plants delineated by an orange-colored frame at the left side of the trays were mock inoculated control plants, which remained healthy throughout the assay. Images were taken at 14 dpi. Control and *P. ultimum* infected plants were maintained under identical greenhouse conditions.

Survival rates for a specific plant genotype were generally consistent or repeatable between infection events. Occasionally, an aberrant survival rate value was observed for a given genotype. Although the infection assay was conducted under "controlled" experimental conditions and efforts were made to maintain the consistency for each step of the experimental procedure, minor variations in plant materials and/or pathogen preparations could contribute to the variable survival rates observed between infection events. Environmental factors, such as fluctuating temperature and/or relative humidity can also present unexpected abiotic stresses which may influence the outcomes of plant-pathogen interactions. Synergistic effects between certain abiotic stress and *P. ultimum* infection may interfere with expression of resistance traits, but the details of their influence are largely unknown. Overall, repeatable and highly consistent results were observed for most of the assayed O3R5 progeny, particularly for the more resistant and susceptible groups as listed in Table 1. The survival rates of all tested genotypes were also analyzed for detecting potential resistance QTLs in collaborator's lab, though no major QTLs were identified.

Table 1. Top ten genotypes from both susceptible and resistant groups

O3R5 genotypes	Total plants assayed (survived)	Times assayed	Range of observed survival rates (%)	average survival rate (%)
#115	122 (24)	10	44-0	**19.5**
#132	57 (18)	4	31-33	**32**
#125	66 (16)	5	36-7	**21.5**
#106	22 (5)	3	33-12	**20**
#121	12 (1)	2	13-0	**6.5**
#47	19 (2)	3	25-0	**11.6**
#80	38 (8)	2	33-10	**21.5**
#34	15 (3)	3	33-0	**19.3**
#4814	25 (7)	3	38-20	**27.6**
#141	38 (9)	3	26-16	**17.3**
#58	49 (43)	5	71-100	**92.4**
#161	57 (51)	5	67-100	**91.6**
#164	91 (77)	6	83-100	**90.5**
G.935	124 (115)	7	86-100	**93**
#172	78 (68)	4	83-100	**87.3**
#173	48 (43)	5	83-100	**89**
#78	67 (61)	6	67-100	**89.4**
#63	38 (35)	2	85-100	**92.5**
#134	19 (17)	2	85-92	**88.5**
#142	42 (32)	3	66-100	**82.3**

Survival rates were scored at 14 dpi. Six plants from the same batch of micropropagation procedure were used as mock-inoculation control which were maintained under the identical conditions to plants inoculated with *P. ultimum*. Numbers of inoculated plants for different O3R5 genotypes varied from 10 to 30 depending on the available plants from tissue culture procedures at the time. At the later stage of the phenotyping study, effort was made to include at least one individual genotype from opposite group to monitor the inoculum preparation, the infection process and/or the presence of other abiotic factors. Average percentage is based on survival rate from each assay.

2. Measured reduction of root and shoot biomasses

In addition to genotype-specific plant survival rates, root and shoot biomass reductions were also measured at 28 dpi to examine the differential impacts of *P. ultimum* root infection on plant growth and

development. By comparing the values of root or shoot fresh weights between mock inoculated control plants and *P. ultimum* inoculated plants, greater reductions in biomass were generally observed for the susceptible genotypes (**Table 2)**. This observation is expected as more severe growth inhibition was often observed for surviving plants from susceptible genotypes as compared to infected plants from resistant genotypes (as shown in Figure 1). On the other hand, substantial reductions in biomass were observed for some resistant genotypes, such as #164 and #172. This observation indicated that although a high percentage of plants manage to be alive at 28 dpi, the growth of these surviving plants can be substantially inhibited due to the continuing influence of *P. ultimum*. In other words, even some of the resistant genotypes that possessed high rates of survival exhibited significant levels of growth inhibition. Therefore, survival rate and biomass reduction are two different aspects of resistance responses, and both parameters should be considered in the evaluation of overall resistance responses for a genotype.

Table 2. Root and shoot biomass reduction due to *P. ultimum* infection

O3R5 genotypes	**Root** biomass (average fresh weight, g)			**Shoot** biomass (average fresh weight, g)		
	Mock inoculation	*P. ultimum* inoculation	Biomass reduction (%)	Mock inoculation	*P. ultimum* inoculation	Biomass reduction (%)
#115 (S)	1.43 ± 0.39	0.71 ± 0.32	**50.3**	0.87 ± 0.05	0.56 ± 0.07	**35.6**
#132 (S)	1.68 ± 0.56	0.85 ± 0.54	**49.4**	1.31 ± 0.36	0.76 ± 0.31	**41.9**
#106 (S)	1.42 ± 0.28	0.42 ± 0.35	**40.8**	1.27 ± 0.27	0.45 ± 0.21	**64.6**
#122 (S)	1.65 ± 0.29	0.99 ± 0.23	**40.0**	1.47 ± 0.34	0.98 ± 0.38	**33.3**
#125 (S)	1.38 ± 0.24	0.86 ± 0.27	**37.7**	1.21 ± 0.02	0.50 ± 0.37	**59.2**
#58 (R)	1.11 ± 0.24	1.05 ± 0.17	**5.4**	0.94 ± 0.08	0.80 ± 0.11	**14.9**
#161 (R)	1.44 ± 0.32	1.35 ± 0.32	**6.2**	1.02 ± 0.12	0.92 ± 0.31	**9.8**
#173 (R)	1.14 ± 0.31	1.03 ± 0.20	**9.6**	0.71 ± 0.10	0.59 ± 0.17	**16.9**
#164 (R)	1.87 ± 0.39	1.35 ± 0.13	**27.8**	1.09 ± 0.07	0.77 ± 0.16	**29.4**
#172 (R)	1.40 ± 0.7	1.11 ± 0.53	**20.7**	1.18 ± 0.11	0.83 ± 0.06	**29.7**

The percentage biomass reduction was calculated by comparing the values of root and shoot fresh weight between the survived plants from *P. ultimum* infection and those of control plants at 28 dpi. Conceivably, the numbers of survived plants varied between resistant and susceptible genotypes from *P. ultimum* infection. R: denotes resistant genotype; S: denotes susceptible genotype.

3. Contrasting patterns of necrosis progression in roots between resistant and susceptible genotypes

The potential mechanisms that function in host resistance at the tissue and cellular levels, which may contribute to the distinct responses to *P. ultimum* infection, were investigated between resistant and susceptible O3R5 genotypes. Using a custom-made small glass box, necrosis progression was monitored continuously along inoculated apple roots under microscope. Based on analysis of serial time-lapsed images, distinctive patterns of necrosis progression were routinely observed along the roots between resistant and susceptible genotypes. As shown in **Figure 2A**, along a section of root system of the susceptible #115 plant, no identifiable infection was observed until 120 hours post inoculation (hpi); then rapid progression of root necrosis, indicated by the yellow or brown coloration from *P. ultimum* infection, spread through entire sections of the root system within 12 hours (120 to 132 hpi). It appeared that no restriction or deterrence existed in root tissues of the susceptible #115, which lead to the swift necrosis progression.

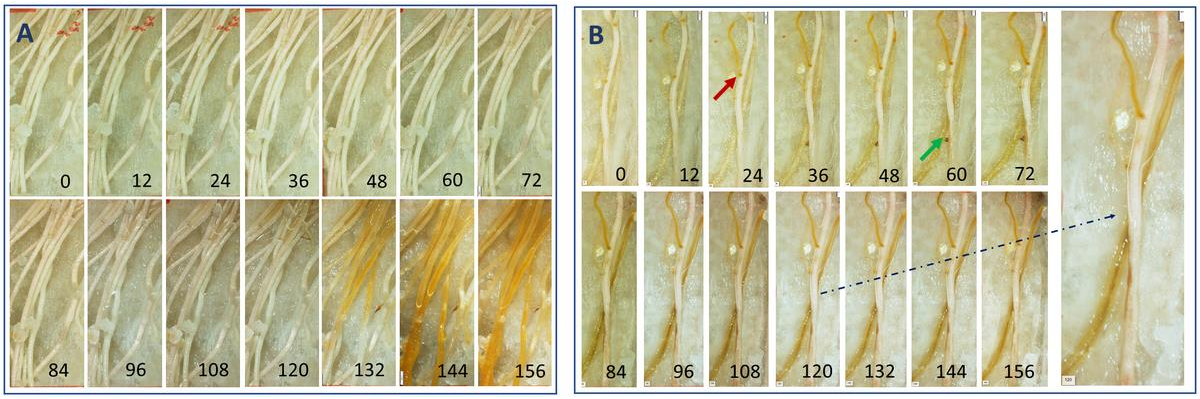

Figure 2. Progression patterns of root necrosis from *P. ultimum* infection. A. Time-lapse images of root necrosis development for the susceptible #115 in response to infection by *P. ultimum*. **B.** Time-lapse images of necrosis progression along the roots of the #161 resistant genotype in response to infection by *P. ultimum*. The number at the bottom of each image denotes the hour post inoculation (hpi).

In contrast, a very different pattern of necrosis progression was frequently associated with the resistant genotypes. As shown in **Figure 2B**, though the necrosis was detected as early as 12 hpi on a newly-emerged lateral root (red arrow on the image for 24 hpi), necrosis initiated from this lateral root seemed to be localized at the junction. A separate necrotic section was observed at 60 dpi from the low section of the roots, as indicated by green arrow, but healthy (white-colored) root tissues were still visible for an extended period of almost 100 hours, from 60 to 156 hpi. Such deterred or delayed necrosis progression was presumably due to the effective resistance responses operating inside the root tissues along roots of the resistant #161 genotype. A close-up image at 120 hpi at the right end of Figure 2B shows a clear "line" or "zone" appear to separate the white-colored healthy section from yellow-brownish necrotic section.

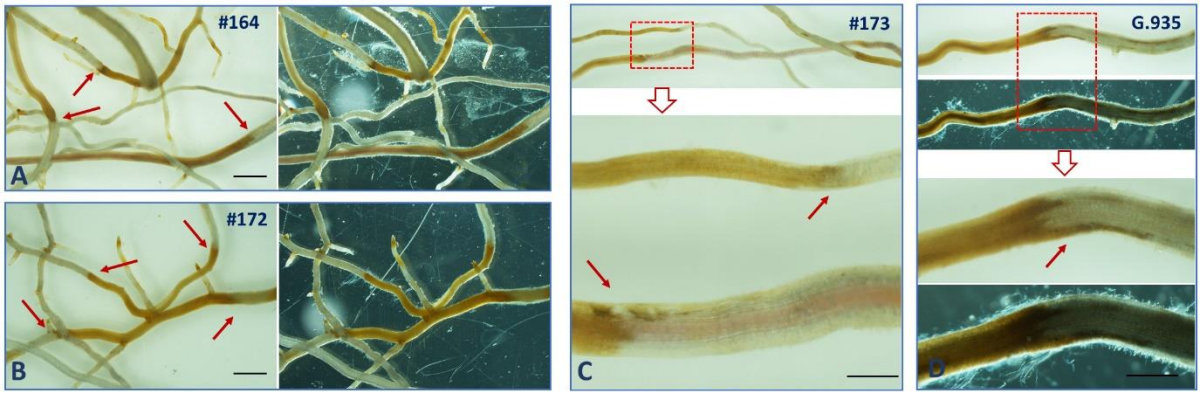

Figure 3. The defined lines separating healthy and necrotic tissues along the infected roots of selected resistant genotypes. The clear and defined "lines" or "zones" can often be observed between sections of necrotic and healthy tissues along the roots of the resistant O3R5 lines. A. Images from infected roots of #164; B. images from infected roots of #172. The same roots sections were documented against both white (left panel) and dark backgrounds (right panel). C. images from infected roots of #173; bottom image is the enlarged section of the top image. D. images from infected roots of G.935; bottom image is the enlarged section from the top image. The bars at the bottom of the image represent 500 µm for A and B; 200 µm for C and D.

Extensive microscopic examination indicated that such a defined "line" or "zone" along the infected roots was more widely associated with other O3R5 resistant genotypes such as #164, #172, #173 and G.935 (Figure 3). The existence of such a "clearly defined line" between healthy and necrotic root tissues strongly suggests that roots of resistant genotypes are capable of deterring the fast-growing *P. ultimum* from inflicting wide-spread necrosis throughout the entire root system. Such delayed necrosis progression could be one of the major factors contributing to the high survival rates of the resistant genotypes. It can be speculated that an active "chemical warfare" operates in the roots of these resistant genotypes towards an invading pathogen. The efficient generation of antimicrobial compounds from effective defense activation could lead to the delayed necrosis progression in the root of resistant genotypes, which in turn provides critical time for regenerating new root branches to compensate the loss of functional root tissue. These defined "zones" were very rarely observed among susceptible genotypes, and never observed from mock inoculated roots.

4. Overlapping resistance response to *R. solani* and that to *P. ultimum*

This part of the experiment was designed to address the question: do apple roots share similar or overlapping mechanisms of resistance to infection by *P. ultimum* and *R. solani*. As shown in **Figure 4**, plant survival rates suggested that considerable comparability exists between the resistance responses to infection by these two pathogens. In other words, those genotypes which were classified as *P. ultimum*-resistant showed a corresponding higher percentage of survival rate from inoculation with *R. solani* AG-5; and all *P. ultimum*-susceptible genotypes except one demonstrated a correspondingly lower survival rate in response to *R. solani* AG-5 inoculation.

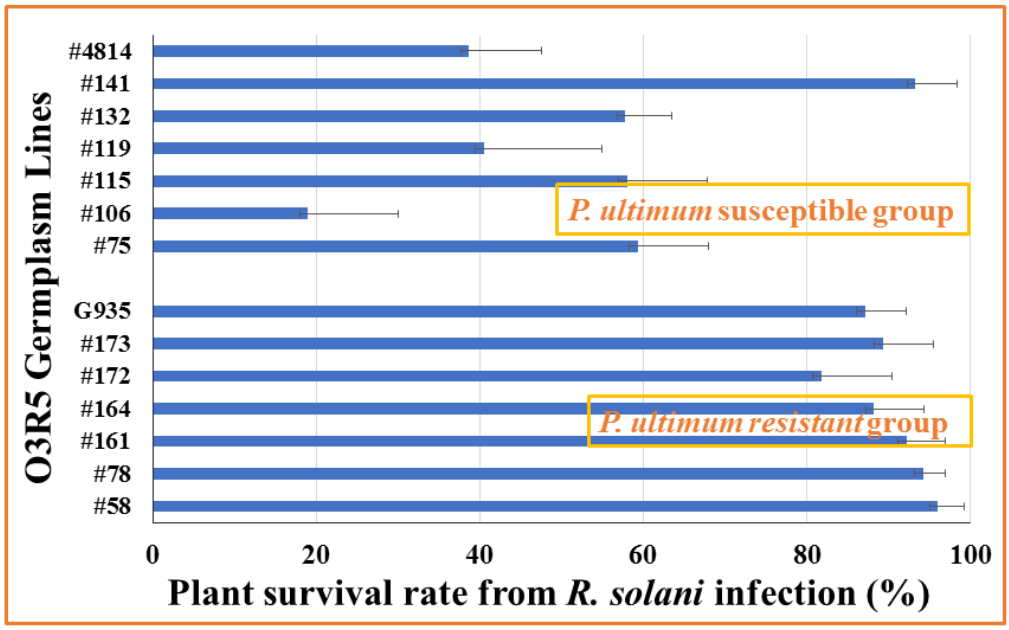

Figure 4. Overlapping resistance responses toward infection by *P. ultimum* or *R. solani*. Results represent the average values from at least two inoculation assays by *R. solani* AG-5. Plant survival rates were scored at 28 dpi. Data were grouped based on the resistance level to *P. ultimum* infection. Individual O3R5 genotypes in the upper group were more susceptible to *P. ultimum* infection, similarly, individual genotypes at lower group were more resistant to *P. ultimum* infection.

It is probably not surprising that comparable resistance responses may exist toward infection from either *P. ultimum* or *R. solani* AG-5 as both are necrotrophic pathogens. Although these two pathogens belong to very different categories (oomycete and fungus), they might exploit some comparable "attacking"

tactics towards apple roots. This observation seems to support the optimistic notion that a certain level of shared resistance mechanisms may exist towards infection by various components within the ARD pathogen complex. Then the level of difficulty could be alleviated in identifying a small set of apple genes to distinguish resistance or susceptibility to ARD. Unlike observations from *P. ultimum* infection, none of the germplasm lines demonstrated single-digit survival rates, this could mean that the *R. solani* AG-5 strain used in this study is less virulent, or less lethal, than the *P. ultimum* strain. However, abundant mycelia were routinely observed from R. *solani* AG-5 inoculated plant roots, suggesting adequate amount of inoculum was applied.

5. Relationship between resistance to *P. ultimum* and extracted *P. penetrans*

The question being asked from this part of experiment was: will those genotypes which showed either resistance or susceptibility to *P. ultimum* infection exhibit a differential effect on populations of the lesion nematode *P. penetrans*. Nematodes were extracted from 5-gram soil samples or 0.5-gram root tissue after plants from selected O3R5 genotypes have grown in the nematode-infested soils for 45 days. As shown in **Figure 5**, it appeared that no identifiable consistency was observed between the resistance to *P. ultimum* and recovered *P. penetrans* numbers. However, disregarding the resistance responses to *P. ultimum* there were considerable variations at recovered nematodes between individual genotypes. For example, #164 demonstrated consistently lower nematode root densities from repeated assays. Another example is obvious differences in term of recovered numbers of nematodes between roots and shoots of G.41. It seems that although resistance to *P. ultimum* infection does not share the trend of nematode density in the soil and root, the genotype-specific variations existed among O3R5 progeny. The detailed mechanisms behind such variation of extracted nematodes numbers are unclear based on the limited data from this pilot experiment. It can be speculated that the number of nematodes may be passively dependent on the genotype-specific availability of leaked nutrients or certain unique chemicals from root system.

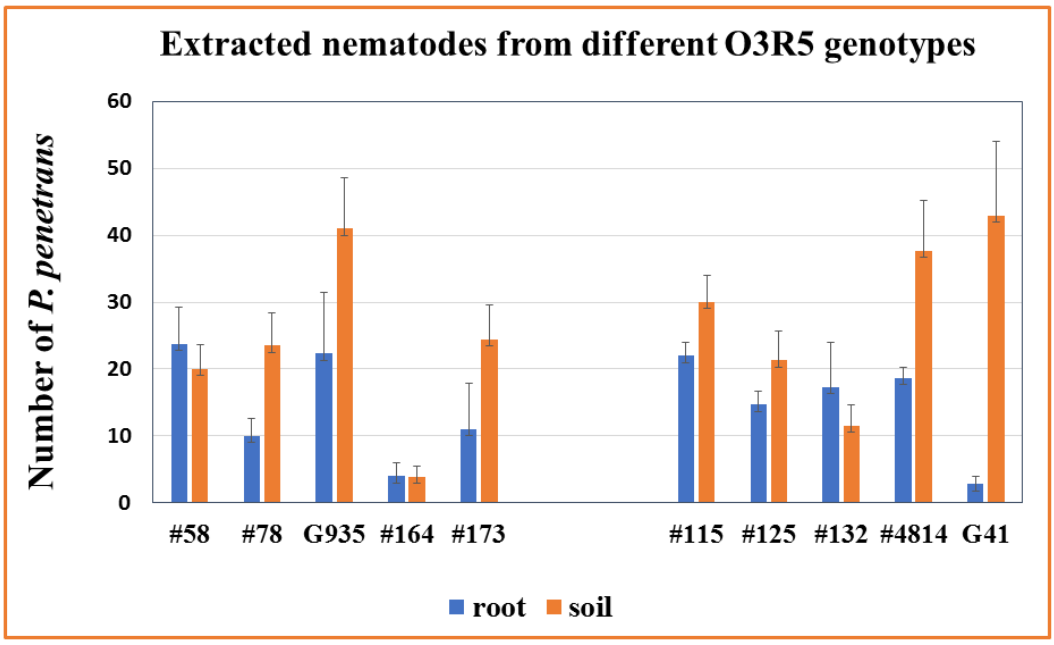

Figure 5. Extracted numbers of *Pratylenchus penetrans* from root and soil per genotypes. Selected O3R5 genotypes with contrasting resistance or susceptibility to *P. ultimum* infection were used to grow in nematode infested soil for 6 weeks. The values represent the average of multiple assays (2-4 times depending on genotypes). Nematodes were extracted from 0.5 g of root tissues and 5 g of soils.

6. Field evaluation of selected genotypes in both fumigated and non-fumigated rows

Field growth responses for selected *P. ultimum*-resistant and *P. ultimum*-susceptible genotypes was carried out at a replant plot at the Columbia View experimental orchard. The values of total plant biomass, were compared between plants growing in fumigated and non-fumigated rows for three months. Based on the data of a short-term (three months) growth responses, those *P. ultimum*-susceptible genotypes showed increased values of biomass in fumigated soil, but only half of *P. ultimum*-resistant genotypes show increased biomass values **(Table 3)**. In other word, those susceptible genotypes suffer more growth inhibition in the non-fumigated row. This preliminary observation seems to support the notion that susceptible genotypes benefit more from soil chemical fumigation, as indicated by the larger increased values of biomass. On the other hand, half of the tested resistant genotypes showed decreased biomass in fumigated soil as indicated by the negative net increase of total biomass values. The mechanism behind this different growth response based on the short-term observation is unknown from this pilot experiment. The possible contributing factors include genotype-specific growth habit or root regeneration patterns under field condition, nutrient utilization efficiency, the ability to overcome heat stress. The age variations between the tested genotypes at the time of being transplanted into soil were considerable for some of them, because of the time needed for generating these plants from tissue culture procedures. Therefore, this part of experiment was certainly a preliminary trial, and a more extensive field evaluation will be needed for more conclusive evidence so that the consistency between greenhouse assay of controlled infection from individual pathogens and overall field performance can be more reliably validated.

Table 3. Growth response of resistant or susceptible O3R5 genotypes to fumigation at replant site

O3R5 genotypes	Phenotype (*P. ultimum*)	total biomass (g); non-fumigated row	total biomass (g); fumigated row	% Net increase (non-F/F)
164#	R	55.7 ±7.4	37.9 ±5.8	-47
173#	R	47.8 ±6.8	43.9 ±7.5	-9
62#	R	189.5 ±19.6	174.3 ±12.8	-9
58#	R	142.0 ±17.3	134.5 ±11.4	-6
B9	S	129.1 ±15.6	127.5 ±13.7	-1
135#	R	89.6 ±11.2	109.2 ±9.9	18
142#	R	67.0 ±8.8	89.7 ±8.1	25
M9	S	259.7 ±22.1	357.9 ±28.3	27
G935	R	84.6 ±9.9	131.4 ±17.4	36
75#	S	79.4 ±11.4	123.6 ±8.4	36
161#	R	82.6 ±7.7	148.3 ±13.2	44
125#	S	31.7 ±5.8	112.2 ±10.1	72

Values represented the average of measured total biomass from all survived plants (up to 5) for each genotype; F: plants grown in fumigated row, non-F, plant grown in non-fumigated row. Designation of R (resistant) or S (susceptible) phenotypes was based on the result of greenhouse infection assay *by P. ultimum*. Fumigant (Telone C-17) was applied by deep (18 inches) untarp broadcast at the rate of 30 gallon per acre on May 25, 2016 by Custom Orchard Fumigation. Plants were planted on late June 2016 and harvested in early Oct for a growing period of more than three months at Columbia View replant site.

EXECUTIVE SUMMARY

Reliable phenotypes are a prerequisite for the conduct of careful molecular and genetic studies concerning the biology of interest. Apple rootstock germplasm with stable resistance traits (and susceptibility) are essential for elucidating the underlying molecular mechanisms. Until this study, a standardized phenotyping protocol for systematic and quantified analysis of apple root resistance responses had been lacking. Using our established phenotyping protocols, more than 60 individuals from O3R5 progeny have been evaluated for their detailed resistance responses to three representative ARD pathogens, i.e. *Pythium ultimum* (oomycete), *Rhizoctonia solani* (fungus) and *Pratylenchus penetrans* (nematode). For the primarily focused pathogen of *P. ultimum*, multiple genotypes with either highly resistant or highly susceptible resistance responses have been identified based on repeated infection assays. Their differential resistance responses were demonstrated by plant survival rates, reduction of root and shoot biomasses, as well as the microscopic features of root tissue necrosis patterns. While substantial overlap was observed between the resistance responses to *P. ultimum* and *R. solani* AG-5, no relationship can be derived between resistance to *P. ultimum* and the recovered nematode *P. penetrans* for a given O3R5 genotype. Preliminary field evaluation at the Columbia View orchard replant site seemed to suggest that the susceptible genotypes (which were based on *P. ultimum* infection assay in greenhouse) benefit more from fumigation than those resistant genotypes do. However, more extensive test with long-term field evaluation is needed. The available resistance phenotyping dataset was also analyzed for detecting potential resistance QTLs in collaborator's lab, though no major QTLs were identified suggesting complex genetics behind the observed resistance responses. This phenotype dataset will be converged with the results of our recently identified candidate apple genes from two transcriptome analyses on apple root defense responses to *P. ultimum* infection. These carefully phenotyped apple rootstock germplasms are pivotal for associating specific apple genes with observed resistance traits. It is worthy to note that the implementation of an in-house micropropagation procedure enabled us to overcome the unique obstacle for studying apple root resistance responses. Though it is a tedious and time-consuming process, the constant supply of uniform apple plants of defined genetic background, equivalent age, and non-contaminated root tissues is fundamental for the high-quality apple root resistance phenotypes. In summary, this study is the first careful and systematic effort to dissect genotype-specific apple root resistance responses to multiple ARD pathogens under controlled experimental conditions. This dataset of apple root resistance phenotype is the necessary step towards maximized exploitation of host resistance in managing ARD in the future. Progress in defining apple root resistance phenotypes from the current study were aligned closely with sustainability and profitability of Washington State apple industry.