Journal of
Plant Pathology & Microbiology
ISSN: 2157-7471

Zhu et al., J Plant Pathol Microbiol 2018, 9:11
DOI: 10.4172/2157-7471.1000461

Research Article Open Access

Identifying an Elite Panel of Apple Rootstock Germplasm with Contrasting Root Resistance to *Pythium ultimum*

Yanmin Zhu[1]*, Jingxian Zhao[2] and Zhe Zhou[3]

[1]United States Department of Agriculture, Agricultural Research Service, Tree Fruit Research Laboratory, Wenatchee, WA, USA
[2]Hebei Academy of Forestry, Shijiazhuang, Hebei Province P.R. China
[3]Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Science, Zhengzhou, P.R. China

Abstract

Apple replants disease (ARD), incited by a soil borne pathogen complex, is a major obstacle to establishing an economically viable apple orchard at replant sites. The predominant control method is pre-plant chemical fumigation of orchard soil, which is expensive and comes with environmental and regulatory concerns. To maximize the exploitation of host resistance for ARD management, high quality resistance phenotypes in apple roots to ARD pathogen infection are required for elucidating the underlying resistance mechanisms. In this study, root resistance responses to *Pythium ultimum* infection were systematically evaluated among the 'Ottawa 3' × 'Robusta 5' (O3R5) F1 progeny. Tissue culture-based micro propagation was employed to generate genetically-defined and age-equivalent apple plants for repeated infection assays. A wide range of plant survival rates were observed, with fewer than 30% for the susceptible genotypes and over 80% for the resistant ones. The levels of root and shoot biomass reduction among the surviving plants varied substantially between the most resistant genotypes and the most susceptible genotypes. Contrasting necrosis patterns were demonstrated along the infected roots between resistant and susceptible genotypes using a novel glass-box pot for continuous microscopic observation. Swift necrosis occurred across the entire root system within 24 hours for the susceptible genotypes; in sharp contrast, evidently deterred root necrosis was observed for the resistant genotypes. A well-defined boundary separating healthy and necrotic root tissues were often accompanied with the infected roots of resistant genotypes, while the profuse growth of *P. ultimum* hyphae was specifically associated with the infected roots of susceptible genotypes. The results from this study represented the first comprehensive and detailed effort undertaken to define the genotype-specific resistance responses in apple roots as they are challenged by a soil-borne pathogen.

Keywords: Apple rootstock; Root resistance responses; *Pythium ultimum*; Resistance traits; Necrosis patterns; Plant micropropagation; Tissue culture

Introduction

Apple (*Malus* × *domestica* Borkh.), a member of the Rosaceae family and Maloideae subfamily, is one of the most popular perennial tree fruits in temperate regions around the world [1]. Apple replant disease (ARD), or replant syndrome, refers to stunted growth or death of newly planted trees at a replant site, where apple or closely related tree species have been previously cultivated. ARD can be a serious obstacle to the establishment of economically viable orchards. The causal agents of ARD consist of a pathogen complex including multiple species belonging to necrotrophic soilborne oomycetes (*Phytophthora* and *Pythium*) and fungi (*Ilyonectria* and *Rhizoctonia*) [2-4]. Among them, *Pythium ultimum* is known to be one of the primary components within the ARD pathogen complex which has been identified in orchard soils worldwide [3,5,6]. Control of ARD has primarily relied on the pre-plant chemical fumigation of orchard soils with the aim of eradicating ARD pathogens [7]. The use of these broad-spectrum fumigants is under increasing regulatory restriction due to environment and human health concerns. In addition to cost, the effects of fumigation are short-lived, and application of these chemicals is not feasible after orchard establishment [8]. Rotation, fallowing, and other cultural disease control methods are either impractical or ineffective for ARD management [9,10]. Alternative control strategies are also being explored, such as using brassicaceae seed meal amendments to exploit microbial communities in orchard soil to suppress pathogen aggressiveness and promote plant health [8,11,12]. It is well-acknowledged that development and deployment of resistant or tolerant rootstocks offers a cost-effective, ecologically friendly and durable approach for ARD management. However, conventional breeding for apple root resistance to soilborne pathogens is a long-term and resource-demanding endeavour [13,14]. Effective screening methods for ARD-resistance are not currently available. Genetics-informed breeding, such as use of predictive DNA markers, promises to enhance precision and efficiency for early selection of desired traits [15,16]. The advantages of marker-assisted breeding can only be realized through careful genetic studies, which in turn demand reliable resistance phenotypes in apple roots [17-19]. Because of their hidden nature and often miniature statues, phenotyping root resistance responses is more challenging compared to that of aboveground organs [20]. Additional obstacles exist for studying resistance responses in roots of apple or other perennial rosaceae tree crops. The apple genome is known for its high level of heterozygosity and extensive whole-genome duplication [21,22]. More importantly, apple reproduction is self-incompatible or outcrossing [1]. As a result, genetically identical plants cannot be produced by apple seed germination, as each seed represents a unique genetic identity. To define the genotype-specific root resistance responses a plant tissue culture-based micro propagation procedure was implemented, which enabled a continual supply of genetically uniform apple plants of any selected apple rootstock genotypes for repeated infection assays. The objective of this study was to identify a panel of apple rootstock genotypes with reliable root resistance phenotypes in response to *P. ultimum* infection.

***Corresponding author:** Yanmin Zhu, United States Department of Agriculture, Agricultural Research Service, Tree Fruit Research Laboratory, Wenatchee, USA, Tel: (509) 664-2280; E-mail: yanmin.zhu@ars.usda.gov

Received September 21, 2018; **Accepted** November 19, 2018; **Published** November 23, 2018

Citation: Zhu Y, Zhao J, Zhou Z (2018) Identifying an Elite Panel of Apple Rootstock Germplasm with Contrasting Root Resistance to *Pythium ultimum*. J Plant Pathol Microbiol 9: 461. doi: 10.4172/2157-7471.1000461

The streamlined phenotyping protocol and the identified genotypes with distinctive root resistance traits can be utilized for future genetic and biochemical analysis to elucidate the resistance mechanisms, and identify specific genes associated with apple root resistance or susceptibility.

Materials and Methods

Plant materials

The 'Ottawa 3' × 'Robusta 5' (O3R5) apple rootstock population was developed in mid-1970s. Both parents, 'Ottawa 3' and 'Robusta 5', are with strong linage of wild apple germplasm [23,24]. The progeny of this cross is known for segregating multiple agronomical traits including dwarfism and resistance to apple fire blight and powdery mildew [16,25,26]. An ARD tolerant germplasm G.935® was originally selected from O3R5 cross population [27], indicating the segregation for ARD resistance. Therefore, O3R5 F1 progeny was chosen for this experiment to phenotyping the root resistance traits to *P. ultimum* inoculation under controlled experimental conditions. Over 90 of O3R5 genotypes were available for this phenotyping effort. Six-year old trees were kept at an experimental orchard for long-term study, which also served as the source of tissue culture based micro propagation of genetically uniform apple plants.

Plant micropropagation and in-soil acclimation of root system

Apple plants with equivalent ages were produced by tissue culture-based micro-propagation procedures as described previously [28]. Briefly, 4-6 weeks are required for shoot propagation and 4 weeks for root elongation. After a sufficient root system has been reached, plants were transferred to pots containing autoclaved Sunshine™ potting mix (SUN GRO Horticulture Ltd, Bellevue, WA) for one week of in-soil acclimation before pathogen infection assays. The step of in-soil acclimation for tissue culture generated root system is a critical requirement in term of allowing further differentiation of root tissues to fully express the inherent resistance traits under pathogenic pressure. To minimize transplanting effects on plants, especially for roots from culture medium to potting soils, a transparent 7" Vented Humidity Dome (Greenhouse Megastore, Danville, IL) was used to cover the flat tray holding the pots for retaining humidity. The temperature in the growth room was approximately 22 ± 1°C at night and 25 ± 1°C during day time with 12 hr light/12 hr dark photoperiod.

Inoculum preparation and inoculation of roots

Inoculum of *Pythium ultimum* was prepared as previously described [28]. The *P. ultimum* isolate used in this study was originally recovered from the roots of 'Gala'/M26 apple grown at Moxee, WA, USA. The inoculum of *P. ultimum* was prepared by cultivating in potato-carrot broth (20 g of carrots and 20 g of peeled potatoes in one L of water boiled for 30 min) with two drops of wheat germ oil added per L of medium [29]. Briefly, the *P. ultimum* cultures were grown in broth in 9-cm Petri dishes at 22°C for 4-6 weeks. Oospores and mycelium from the resultant mat were collected and ground in 0.5% methyl cellulose solution using a household electric blender for 30s. The oospores and hyphal fragments were resuspended in 0.5% methyl cellulose to give a final concentration of approximately 2,000 oospores per mL. The inoculation of seedlings with *P. ultimum* was performed by dipping the root system in the inoculum solution for 5 s. Inoculated plants were immediately transplanted into autoclaved Sunshine™ potting mix in 4" pots and thoroughly watered. Control plants were mock-inoculated

O3R5 genotypes	Total plants assayed (survived)	Times assayed	Range of observed survival rates (%)	Average survival rate (%)
#115	76 (14)	5	0-44	18.4
#132	78 (15)	5	5-33	19.2
#47	63 (16)	5	0-25	25.4
#106	67 (16)	5	12-33	23.9
#121	72 (12)	4	0-23	16.7
#58	103 (90)	6	71-100	87.4
#161	72 (65)	5	67-100	90.3
#164	69 (66)	5	83-100	95.7
#78	92 (78)	5	67-100	84.8
#63	81 (70)	4	85-100	86.4

Table 1: Selected *O3R5* genotypes demonstrating distinctive survival rates.

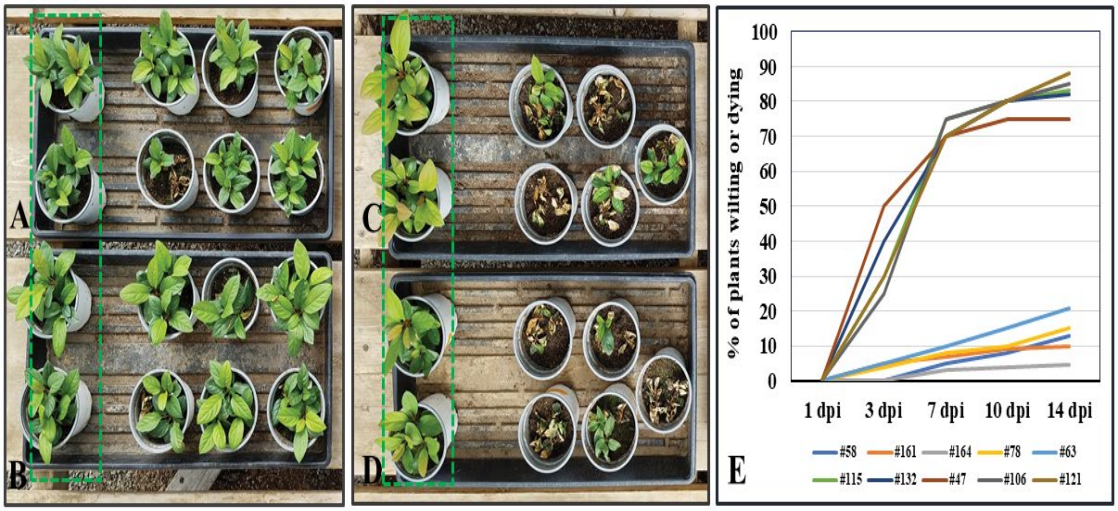

Figure 1: Distinctive survival rates among O3R5 progeny in response to infection by *Pythium ultimum*. **A.** The resistant genotypes O3R5-#161; **B.** The resistant genotype O3R5-#58; **C**. The susceptible genotype O3R5-#115; **D**. The susceptible genotype O3R5-#132. Those plants at the left column (highlighted in a green frame) were mock-inoculation control for each genotype. Plants for all these four genotypes were inoculated simultaneously using the same inoculum preparation. Control and *P. ultimum* infected plants were maintained under identical growth conditions. Image was taken at 14 dpi. **E.** The representing patterns of the timeline of manifestation of wilting symptom and/or mortality after pathogen inoculation.

O3R5 genotypes	Root biomass (average fresh weight, g)			
	Mock-inoculation	*P. ultimum* inoculation	Biomass reduction (%)	*P* value
#115 (S)	1.19[a] ± 0.39	0.71[b] ± 0.32	40.3	0.007
#132 (S)	1.04[a] ± 0.56	0.75[b] ± 0.54	27.9	0.01
#106 (S)	1.12[a] ± 0.28	0.82[b] ± 0.35	26.8	0.03
#47 (S)	0.95[a] ± 0.19	0.73[b] ± 0.15	23.2	0.04
#121 (S)	1.13 ± 0.23	0.89[b] ± 0.17	21.2	0.03
#58 (R)	0.89[a] ± 0.21	0.91[a] ± 0.17	-2.2	0.86
#161 (R)	1.14[a] ± 0.32	1.05[a] ± 0.32	6.2	0.09
#63 (R)	1.03[a] ± 0.19	0.91[b] ± 0.27	11.7	0.04
#78 (R)	0.72[a] ± 0.21	0.64[a] ± 0.22	11.1	0.25
#164 (R)	0.81[a] ± 0.16	0.96[a] ± 1.52	-1.19	0.68
O3R5 genotypes	**Shoot biomass (average fresh weight, g)**			
	Mock-inoculation	***P. ultimum* inoculation**	**Biomass reduction (%)**	***p* value**
#115 (S)	0.87[a] ± 0.15	0.56[b] ± 0.07	35.6	1.2E-05
#132 (S)	1.21[a] ± 0.36	0.93[b] ± 0.31	23.1	0.02
#106 (S)	1.27[a] ± 0.27	0.85[b] ± 0.21	33.1	0.004
#47 (S)	0.97[a] ± 0.37	0.66[b] ± 0.24	32.0	0.04
#121 (S)	1.23[a] ± 0.24	0.78[b] ± 0.19	36.6	0.01
#58 (R)	1.03[a] ± 0.08	1.05[a] ± 0.11	-1.9	0.08
#161 (R)	1.02[a] ± 0.12	0.92[a] ± 0.31	9.8	0.06
#63 (R)	1.29[a] ± 0.32	1.08[b] ± 0.21	16.3	0.03
#78 (R)	0.94[a] ± 0.21	0.76[b] ± 0.16	19.1	0.0002
#164 (R)	1.22[a] ± 0.19	0.91[b] ± 0.18	25.4	0.0001

The percentage of biomass reduction was calculated by comparing the means of root or shoot fresh weigh between the surviving plants from *P. ultimum* infection and those of control plants measured at 28 dpi. R: denotes resistant genotype; S: denotes susceptible genotype. Means for treated and control treatments with the same letter do not differ according to *t*-test, based on the criteria of *P* (probability) values < 0.05.

Table 2: Root and shoot biomass reduction between genotypes with higher or lower survival rates.

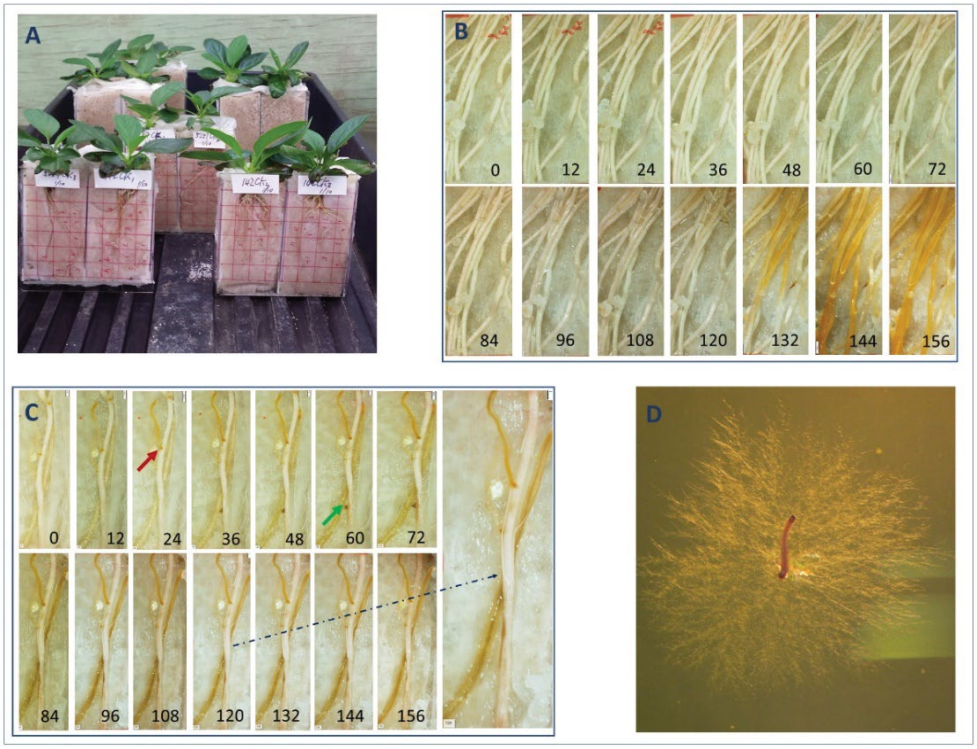

Figure 2: Genotype-specific patterns of tissue necrosis progression due to *Pythium ultimum* infection. **A**. The set-up of glass-box with roots exposed by aligning against the glass plate, the labelled grid for facilitating the localization of root section for continuous observation. **B.** Time-lapse images of root necrosis progression for a susceptible genotype O3R5-#115 in response to infection by *P. ultimum*. **C.** Time-lapse images of necrosis progression for a resistant genotype O3R5-#161 in response to infection by *P. ultimum*. In both C and D, the number at the bottom of each image denotes the hour post inoculation (hpi). **D.** Image of *P. ultimum* hyphae growing out from a necrotic root segment after incubation on a PSSM agar plate.

with 0.5% methyl cellulose solution and maintained similarly to the *P. ultimum* inoculated plants. All plants were grown as triplicates per pot and maintained in an environmental growth room under a 12 hr light/12 hr dark cycles.

Phenotypic assessment of plant survival rates and biomass reduction

For plant survival rate and biomass reduction analysis, inoculated plants were allowed to grow for four weeks in autoclaved potting mix. Plant survival rate for each genotype was recoded at 3, 7, 10, 14 and 28 dpi (days post inoculation). Plant biomass reductions were determined at 28 dpi by measuring root or shoot fresh weights between mock-inoculated control plant and *P. ultimum* infected but surviving plants. For measuring root and shoot biomass, plants were carefully removed from pots, and soil medium was gently rinsed off root tissues under tap water. Individual plants were wrapped in moist paper towels before

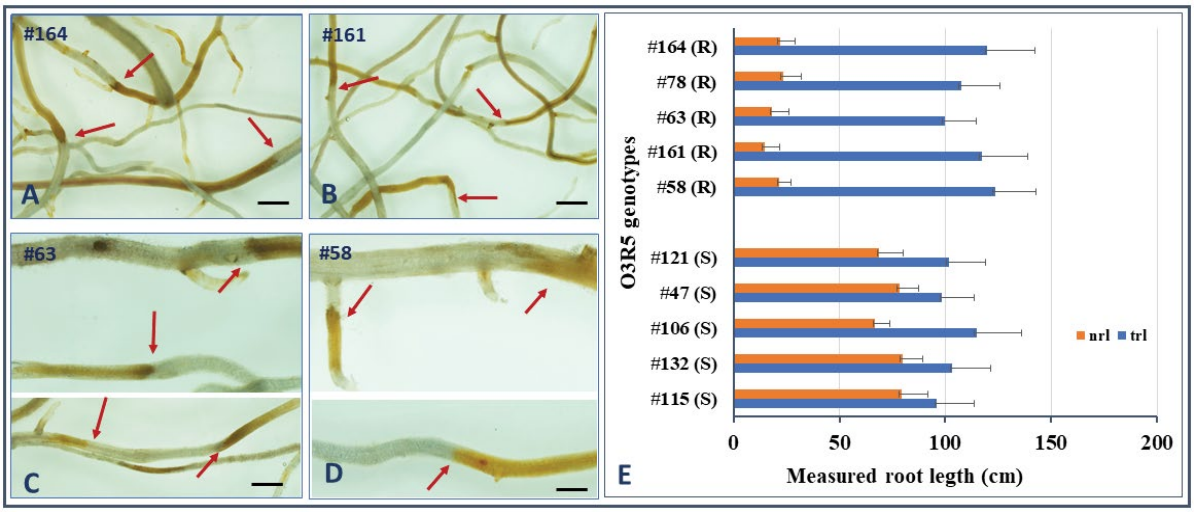

Figure 3: The defined boundaries separating healthy and necrotic tissues along the infected roots of resistant genotypes. The clear and defined "lines" or "zones" were often observed at 48 to 120 hpi along the infected roots of the resistant O3R5 lines. **A.** Images from infected roots of #164; **B.** images from infected roots of #161; **C.** images from infected roots of #63; **D.** images from infected roots of #58. The bars on the bottom images represent 500 µm. **E.** Measured values of necrotic sections and total root length for selected O3R5 genotypes. Segments from the entire root system were photographed and the root length was measured using publicly available software ImageJ (https://imagej.nih.gov/ij/). The legend of nrl denotes for "necrotic root lengths"; and trl for "total root length". The measured values represent the means from infection assays; each infection includes at least 5 infected plants.

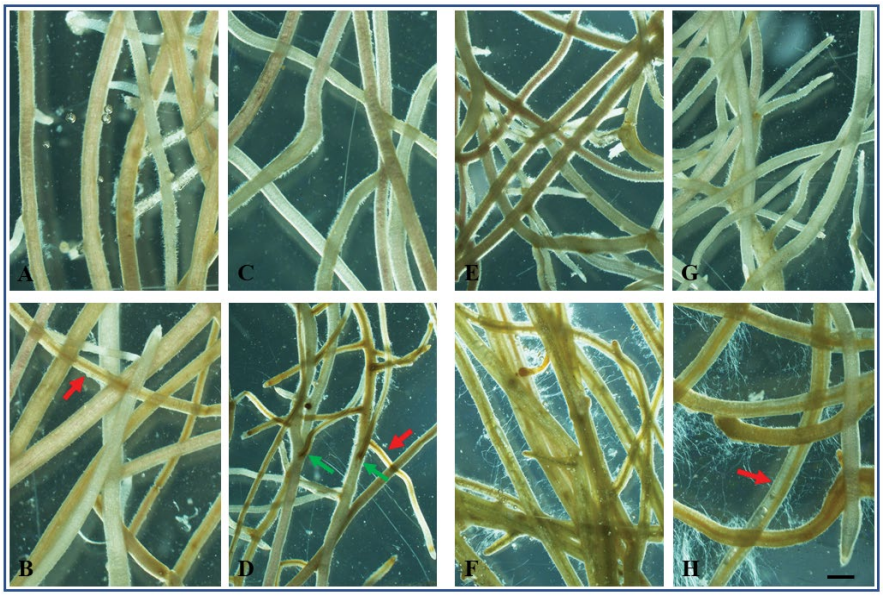

Figure 4: Microscopic features related to tissue necrosis and pathogen hyphae growth between resistance and susceptible genotypes at 48 hpi. Images of **A** and **B** were from a more resistant genotype of O3R5-#58; **C** and **D** were from a more resistant genotype of O3R5-#63; **E** and **F** were from a more susceptible genotype of O3R5-#106; and **G** and **H** were from a more susceptible genotype of O3R5-#47. The images at the upper row (**A, C, E** and **G**), represented the control treatment of mock-inoculated roots; and **B, D, F** and **H** at the lower row were the images for roots inoculated with *P. ultimum* for corresponding genotypes. Roots for at least three plants were examined for each genotype, and observations were repeated for two independent inoculation events. The representing images were taken with the same power of magnification; the bar in image H represents 400 µm.

weighing the shoot and root biomass separately. Separate experiments were carried out to determine the ratio between total root length and necrotic root length. Root branches were placed in Petri dishes and photographed using a Canon D35 camera. The acquired images were processed using publicly available software ImageJ (https://imagej.nih.gov/ij/) for measuring total and necrotic root length. Necrotic sections were primarily based on the presence of yellow to brownish root coloration associated with necrotic root tissues.

Microscopic observation of genotype-specific necrosis patterns

For microscopic observation, autoclaved soil medium consisting of construction sands, vermiculite, and perlite in a ratio of 1:1:1 was used to fill the glass box or pot. Plants were carefully excavated from the soil to minimize mechanical damage to the roots at designated time points. Residual soil along the root branches was gently removed under running tap water. Roots for both mock-inoculated control and *P. ultimum* inoculated plants were kept separately in 100-mL beakers filled with water until microscopic examination within two hours. Individual root branches were separated from each other, and a glass slide was used to hold the root in petri dish filled with autoclaved water (for minimized air bubble formation). A minimum of six plants per genotype and treatment were examined with the assistance of a dissecting microscope (Olympus SXZ12). For continuous observation of necrosis progression along the infected roots, a small glass box (2.5 × 7.5 × 10 cm) was used to hold each plant, aligning the root system against the glass plate and separating roots from soil substrate by a sheet of paper towel. The glass box was wrapped with aluminium foil to minimize unnecessary light exposure to root system until microscopic observation. Grid lines on the glass box were used to facilitate locating and tracking the specific sections of a root system. Images of root tissues were obtained using a DP73 digital camera installed on an Olympus SXZ12 dissecting microscope and the associated software suite of celSense (Olympus, Center Valley, PA). Digital images were slightly modified such as resizing, cropping and adjusting overall brightness of obtained images using a publicly available software Faststone Image Viewer 5.5 (www.faststone.org).

Statistical analysis

All measure values were three biological replicates. Data were analyzed using ANOVA followed by paired or unpaired Student's *t* test (Microsoft Excel). Differences of means with $p < 0.05$ were considered significant.

Results and Discussion

Widely-distributed survival rates among O3R5 genotypes to *P. ultimum* inoculation

Based on repeated infection assays, a wide-spectrum of plant survival rates was observed across the tested sixty-three O3R5 genotypes in response to *P. ultimum* inoculation. Plant survival rates were recorded at 28 dpi (days post inoculation), and those genotypes with average survival rates above 80% were designated "resistant", and those with lower than 30% survival rates were considered as "susceptible". Most genotypes fell into the category with mediocre level of survival rates, as expected, between these two established thresholds. The top five most resistant and susceptible genotypes are listed in Table 1. As examples, two resistant genotypes (O3R5-#161 at top and O3R5-#58 at bottom show almost 100% survival rate without visible growth inhibition (Figures 1A and 1B). In contrast, less than 30% of plants from two susceptible genotypes (O3R5-#115 at top and O3R5-#132 at bottom) (Figures 1C and 1D) are survived but with easily identifiable growth inhibition for the surviving plants at 14 dpi. The typical timeline for initially discernable wilting symptoms on leaf can be identified as early as 3 dpi for the more susceptible genotypes (Figure 1E). The percentage of plants with wilting and dying symptoms was generally stabilized at 7dpi, with slight changes between 7 and 14 dpi. For a given genotype, plant survival rates were mostly consistent between 4-6 independent infection events, indicating the applicability of this infection protocol among the tested apple rootstock germplasm. Disease tolerance of apple rootstock selections is traditionally evaluated under field conditions using one-year old bareroot trees, or so-called "rootstock sticks", from stool-bed propagation in commercial nurseries [14,30]. The indirect physiological parameters including tree height, stem diameter, and multi-year accumulated fruit yield were used to infer ARD tolerance, without the knowledge of intrinsic apple root responses. While this is a practical and valuable approach for evaluating field performance of a rootstock, it is probably not an optimized methodology for acquiring detailed and reproducible root resistance phenotypes for subsequent molecular or genetic analyses. Multiple non-genetic factors can influence the expression of resistance phenotypes such as soil types, soil nutrient and microclimate conditions associated with a specific orchard [13]. The availability of these "rootstock sticks" is often limited to only the elite commercial varieties and for a narrow time window. Probably, the most important consideration regarding molecular research is that the exposure of root system to other untargeted soils microbes or unintended abiotic factors prior to inoculation by ARD pathogens may complicate the expression of its genotype-specific resistance responses. The uneven root systems between individual trees can also tamper with the careful examination of genotype-specific resistance responses. Due to the lack of consistent availability of uniform plants (and root systems), the genotype-specific resistance responses in apple roots to infection by ARD pathogens have not been carefully investigated [28]. The primary objective here is to obtain the reliable or reproducible resistance phenotypes to a targeted ARD pathogen under controlled environment, whether they will have a consistent resistance performance under field condition requires further investigation. The constant availability of these uniform apple plants through tissue culture-based micropropagation indisputably overcomes a major hurdle for the systematic phenotyping effort on apple root resistance traits. Specifically, it was demonstrated that the applied pathogen inoculum level can effectively discriminate the level of resistance responses among tested O3R5 genotypes. Plant survival rates were scored at 28 dpi. Six randomly selected plants for the same genotype were set as the mock-inoculation control in each infection event. Both mock-inoculated and *P. ultimum* inoculated plants were maintained under identical conditions. Numbers of inoculated plants for an O3R5 genotype varied from 12 to 36 depending on the output from tissue culture procedures at the time. Average survival rate for a specific genotype was calculated based on the total and survived plants from 4-6 independent inoculation events.

The levels of biomass reduction partially associated with plant survival rates

The genotype-specificity of plant growth inhibition, in addition to the observed plant survival rates, was investigated by quantifying and comparing the root and shoots biomasses between mock-inoculated and *P. ultimum*-inoculated plants at 28 dpi. Not surprisingly, the susceptible genotypes often demonstrated a greater percentage of biomass reductions for both root and shoot tissues (Table 2). O3R5-#115 and O3R5-#132 suffered 30%-40% of biomass reduction for both root and shoot at 28 dpi. The reduced plant size due to *P. ultimum*

infection was often visibly identifiable as early as 14 dpi for the survived plants (as shown in Figure 1B), comparing to that of mock-inoculated control plants. In contrast, resistant genotypes suffered a less dramatic biomass reduction in general. Root and shoot biomasses suffered a minimal impact (less than 10%) due to pathogen infection for O3R5-#58 and O3R5-#161. However, the shoot biomass for some of the resistant genotypes, such as O3R5-#164 and O3R5-#78, exhibited statistically significant reduction even the values for root biomass reduction were not significantly different, as compared to the mock-inoculation controls. Therefore, the level of biomass reduction for a specific genotype adds another critical evaluation for assessing the overall root resistance responses beside plant survival rate. The levels of biomass reduction revealed another quantifiable aspect of apple rootstock resistance responses in addition to the genotype-specific plant survival rates. Although some resistant genotypes such as O3R5-#63 and O3R5-#78 exhibited higher than 80% plant survival rates, the reduced plant sizes among survived plants due to *P. ultimum* inoculation were often easily observable towards the end of the assays at 28 dpi. Furthermore, the reduction of root biomass for the resistance genotypes, such as O3R5-#164 and O3R5-#78 were not statistically significant, but the values for shoot biomass demonstrated the significant reduction due to *P. ultimum* infection. This later observation seemed to suggest that the physiological function of infected roots may have been considerably compromised even though the statistically significant difference ($p \geq 0.05$) in root biomass reduction was not detected in the assay. In other words, root size or root mass of the infected roots may be still similar to those in mock-inoculated roots in weight, but it is likely the partially affected physiological function failed to meet the need for the growth of aboveground tissues. Multi-dimensional evaluation of root resistance response is needed for a reliable and appropriate assessment for overall genotype-specific resistance levels.

Contrasting patterns of necrosis progression along infected roots between resistant and susceptible O3R5 genotypes

The possible cellular mechanisms which contribute to the observed variations of survival rate and biomass reduction in response to *P. ultimum* infection remain unknown. Using a custom-made small glass box (2.5 × 7.5 × 10 cm) (Figure 2A), genotype-specific root necrosis progression from *P. ultimum* infection was monitored and documented with the assistance of a dissecting microscope. The contrasting patterns of necrosis progression between resistant and susceptible genotypes were revealed by analysing the serial images from the same section of *P. ultimum* infected root system. The necrosis progression along the infected roots of O3R5-#115 represent a typical pattern for the susceptible genotypes (Figure 2B). No identifiable necrosis was detected until 120 hours post inoculation (hpi) within this section of the root system; then a rapid expansion of necrotic tissues occurred from 120 to 132 hpi across the entire section of the root system, as indicated by the appearance of the yellow to brownish coloration. On the other hand, along the roots of a resistant genotypes O3R5-#161 a distinctive pattern of necrosis progression was demonstrated. As shown in Figure 2C, though the initiation of necrosis can be detected as early as 12 hpi on a newly-emerged root (red arrow at 24 hpi image) of the resistant genotype O3R5-#161, tissue necrosis appeared to be limited to the junction with the older root during the entire observation period. A separate necrotic section was observed at 60 hpi from the lower side of the roots (green arrow in 60-hpi image), but the healthy or white-colored root tissues remained visible for an extended period of almost 100 hours, from 60 to 156 hpi. A close-up image at 120 hpi at the far right of Figure 2B showed a defined "line" or "zone", which appeared to serve as a barrier, separating the white-colored healthy section from yellow to brownish necrotic sections. The presence of *P. ultimum* hyphae was observed from a segment of necrotic root after 24-hour incubation on the semi-selective PSSM medium (Figure 2D). Due to the hidden nature of plant root system, the symptom development due to soilborne pathogen infection is inherently more challenging to monitor as compared to that for the aboveground organs. It is conceivable that the host-pathogen interactions at tissue and cellular levels ultimately determine the distinctive plant mortality and the levels of stunted growth among these O3R5 genotypes in response to *P. ultimum* infection. These observed necrosis progression patterns provide the important insight for elucidating the underlying cellular mechanisms leading to resistance or susceptibility. These observations represent the first detailed documentation on necrosis development along infected apple roots in response to a soilborne pathogen. The deterred or delayed necrosis progression along the roots of the resistant O3R5-#161 indicated the existence of a functional or effective cellular defense mechanism. Incidentally, the early detection of symptom at this specific section of the root system for the resistant genotype of O3R5-#161 provided a wider time window for monitoring deterred necrosis process. In contrast, even though the detection of the necrosis initiation along the root section of the susceptible O3R5-#115 was late at 120 hpi, the swift expansion of necrotic tissues throughout the entire section of root system clearly demonstrated the inability to confine the pathogen progression once infection is initiated. The use of these small glass-box pots and the available small plants provided, for the first time, the unique opportunity for continuous and direct microscopic observation on these tiny apple roots, in a non-disruptive and non-destructive manner with minimized invasiveness.

The defined boundaries separating necrotic and healthy sections along infected roots mostly associated with the resistant genotypes

A more careful examination indicated that the well-defined boundaries, which separate the yellow brownish necrotic tissues from the white-colored healthy sections, appeared to be commonly associated with the infected roots among the resistant O3R5 genotypes. Such boundaries ("lines" or "zones") along the infected roots were often detected for most resistant genotypes from 2 to 7 dpi (Figures 3A and 3D). These defined boundaries likely functioned as a deterrence or compartmentalization on pathogen progression; and therefore, prohibited the extensive expansion of necrosis into the other parts of the entire root system. As a result, the formation of such distinctive boundaries contributed to the distinctive values of measuring necrotic root lengths (nrl) at 72 hpi, in relation to the total root length (trl) (Figure 3E) between resistant and susceptible O3R5 genotypes. The similar boundaries were rarely observed along the infected roots of the susceptible genotypes. No such features were found from roots of mock-inoculated control plants. Microscope-assisted observations offered some interesting details on the nature of interaction between apple root tissue and invading *P. ultimum*. The presence of such well-defined boundaries which separate healthy and necrotic sections suggests that apple root tissues of resistant genotypes are able to restrict or arrest the aggressiveness of the fast-growing *P. ultimum*. The constrained pathogen aggressiveness likely prevented necrosis spreading out to the entire root system. The sites where boundaries formed could be the front of battle between apple root cells and the invading pathogen. Along these young apple feeder roots, physical barriers such as cuticle layer or secondary cell wall barely exist to ward off pathogen invasion. Therefore, the efficient synthesis and timely delivery of antimicrobial compounds to infection sites probably represent the primary defense responses [31-33]. From the point of plant survivability, even the

temporary or partial deterrence of pathogen aggressiveness could offer the critical time window for regenerating new roots. Conversely, lack of efficient cellular defines output permits invading pathogens to quickly overpower the root defines system. These observed boundaries were not observed on each root branch within the inoculated root system, and the frequency and locations of their formation is not predictable between inoculation events. Nevertheless, it is highly possible that these boundaries or barriers contributed to the deterred necrosis progression and the observed higher survival rates among the resistant O3R5 genotypes.

The profuse growth of pathogen hyphae commonly associated with infected roots of susceptible genotypes

Previous pilot phenotyping survey on apple root resistance and recent transcriptase analyses on apple root-*P. ultimum* interactions [28,34,35] indicate that the timepoint at 48 hpi was a critical stage for this pathosystem. Careful microscopic examinations on the infected roots, against the dark viewing background, revealed that the profuse pathogen hyphae growth appeared to be specifically associated with the susceptible O3R5 genotypes. As shown in Figure 4, all mock-inoculated roots as control for four genotypes remained healthy at 48 hpi, as indicated by the white color of root branches and the intact appearance of root tissues (i.e., none-transparent root cortex tissues) for all tested genotypes (Figures 4A, 4C, 4E and 4G). For *P. ultimum* inoculated roots, contrasting images were observed between the resistant and susceptible genotypes. For two resistant genotypes, O3R5-#58 and O3R5-#161, only a few root branches were identified with the signs of tissue necrosis, i.e., yellow to brownish coloration with identifiable transparency within root cortex tissues (Figures 4B and 4D; red arrows). The large part of the root systems appeared to remain healthy with white-colored roots and intact (or non-transparent) tissues, similar to those of mock-inoculated roots. Features of deterred necrosis expansion (at the junction of root fork) by the formation of defined boundaries were also exhibited (Figure 4D; green arrows). In contrast, almost all root branches became necrotic for two susceptible genotypes of O3R5-#47 and O3R5-#106 (Figures 4F and 4H), as reflected by the yellow to brownish coloration and the severely collapsed tissues for almost all the root branches. The semi-transparent root cortex tissues were easily visible among the necrotic roots (red arrows). The most revealing feature was the profuse growth of *P. ultimum* hyphae along the infected root branches, but only along the infected roots of the susceptible genotypes of O3R5-#47 and O3R5-#106. As a sharp contrast, minimal hyphae growth was observed along the infected roots of two resistant genotypes O3R5-#58 and O3R5-#161 (Figures 4B and 4D). Because young apple feeder roots are tiny, it is difficult to monitor the symptom development in response to pathogen infection. Use of glass-box container allowed the continuing, real-time and non-interrupted observation on the necrosis progression patterns between resistant and susceptible genotypes. However, certain features related to the pathogenesis processes, such as the levels of root tissue disintegration and richness of hyphae growth required a more careful microscopic examination. Here, the microscopic observation against the dark viewing-field revealed that the profuse growth of *P. ultimum* hyphae was specifically associated with the infected roots of susceptible genotypes, but not resistant genotypes. The seemingly unrestricted growth of *P. ultimum* hyphae corroborates the notion of ineffective defense output in roots of the susceptible genotypes. The inability to deter pathogen aggressiveness most likely contributed to the elevated plant mortality for susceptible genotypes. It is clear that various observation methods are needed for the comprehensive and in-depth evaluation of genotype-specific resistance responses, and accurate and reproducible apple root resistance traits.

Conclusion

In the post-genomics era, reliable phenotypic data is often considered as a major operational bottleneck which hinders the realization of genetic potential contributing to agricultural productivity and sustainability [17,20,36]. Careful and meaningful molecular and genetic studies demand the high-quality phenotypes for the biology of interest such as apple root resistance to ARD pathogens. To tackle the more challenging task in phenotyping root resistance for rosaceae tree crops like apple, use of tissue culture micropropagated uniform apple plants enabled the repeated infection assays for evaluating the inherent resistance traits such as genotypes-specific plant survival rates and the levels of biomass reduction. A non-disruptive approach combining glass box pot and dissecting microscope made it possible for continuous observation of necrosis development along infected apple roots for the first time. The common association of the defined boundaries separating healthy and necrotic sections along the infected root of resistant genotypes indicated an effective resistance mechanism. On the other hand, the profuse growth of pathogen hyphae along the infected roots of susceptible genotypes demonstrated the lack of functional cellular defense output and unsuccessful deterrence of pathogen aggressiveness. The streamlined phenotyping protocol provides a valuable tool for careful and systematic analysis of apple root resistance response. The identification of this panel of apple rootstock germplasm with reproducible and contrasting resistance phenotypes is pivotal for the subsequent genetic studies and biochemical analyses with the attempt to unravel the molecular and genetic controls over these observed resistance traits. This report represented the first comprehensive study undertaken to define the inherent resistance traits in apple roots to response to infection by a soilborne pathogen *P. ultimum*. Therefore, the report is a valuable contribution to interactions between plant roots and soilborne pathogen, particularly for the perennial tree fruit crops.

Acknowledgements

The authors thank Melody Saltzgiver, Connor Foli and Edward Valdes for their excellent technical assistance. This work was supported by the research fund from Washington Tree Fruit Research Commission and USDA ARS base fund.

Conflict of Interests

The research described in this manuscript was in part funded by a grant from Washington State Tree Fruit Research Commission. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This funding does not alter the authors' adherence to all polices on sharing data and materials. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Authors Contributions

YZ directed the phenotyping study, collected phenotyping data, conceived the research, designed the experiments, analyzed the results, and wrote the manuscript. JZ and ZZ performed infection assay, raw data collection and microscope observation.

References

1. Janick J, Cummins J, Brown S, Hemmat M (1996) Apples. Fruit Breed 1: 1-77.
2. Jaffee B, Abawi G, Mai W (1982) Role of soil microflora and *Pratylenchus penetrans* in an apple replant disease. Phytopatho 72: 247-251.
3. Jaffee B, Abawi G, Mai W (1982) Fungi associated with roots of apple seedlings grown in soil from an apple replant site. Plant Dis 66: 942-944.
4. Mazzola M (1998) Elucidation of the microbial complex having a causal role

in the development of apple replants disease in Washington. Phytopatho 88: 930-938.

5. Mazzola M (1997) Identification and pathogenicity of *Rhizoctonia* spp. isolated from apple roots and orchard soils. Phytopatho 87: 582-587.
6. Tewoldemedhin YT, Mazzola M, Botha WJ, Spies CF, McLeod A (2011) Characterization of fungi (*Fusarium* and *Rhizoctonia*) and oomycetes (*Phytophthora* and *Pythium*) associated with apple orchards in South Africa. European Journal of Plant Patholo 130: 215-229.
7. Covey RP, Benson NR, Haglund WA (1979) Effect of soil fumigation on the apple replant disease in Washington. Phytopathology, 69: 684-686.
8. Mazzola M, Strauss S (2013) Resilience of orchard replant soils to pathogen re-infestation in response to Brassicaceae seed meal amendment. Asp App Bio 119: 69-77.
9. Okubara PA, Dickman MB, Blechl AE (2014) Molecular and genetic aspects of controlling the soil-borne necrotrophic pathogens Rhizoctonia and Pythium. Plant Sci 228: 61-70.
10. Trout T, Fresno C (2001) Impact of township caps on Telone use in California. See CUE 0003-0028.
11. Mazzola M, Granatstein DM, Elfving DC, Mullinix K (2001) Suppression of specific apple root pathogens by Brassica napus seed meal amendment regardless of glucosinolate content. Phytopatho 91: 673-679.
12. Mazzola M, Hewavitharana SS, Strauss SL (2015) Brassica seed meal soil amendments transform the rhizosphere microbiome and improve apple production through resistance to pathogen reinfestation. Phytopatho 105: 460-469.
13. Cummins JN, Aldwinckle HS (1995) Breeding rootstocks for tree fruit crops. New Zealand Journal of Crop and Horticultural Science 23: 395-402.
14. Fazio G, Robinson TL, Aldwinckle HS (2015) The Geneva apple rootstock breeding program. Plant Breed Rev 39: 379-424.
15. Aldwinckle H, Cummins J, Forsline P, Holleran H, Norelli J, et al. (2000) The new USDA-ARS/Cornell University apple rootstock breeding and evaluation program. In: VII Int Sympo on Orch and Planta Syst 557: 35-40.
16. Fazio G, Aldwinckle H, Robinson T, Wan Y (2008) Implementation of molecular marker technologies in the Apple Rootstock Breeding program in Geneva-challenges and successes. In: IX Int Symposium on Integrating Canopy, Rootstock Env Physio in Orchard Syst 903: 61-68.
17. Fiorani F, Schurr U (2013) Future scenarios for plant phenotyping. Ann Rev Plant bio 64: 267-291.
18. Ogura T, Busch W (2016) Genotypes, networks, phenotypes: moving toward plant systems genetics. Ann Rev Cell and Develop Bio 32: 103-126.
19. Bazakos C, Hanemian M, Trontin C, Jiménez-Gómez JM, Loudet O (2017) New strategies and tools in quantitative genetics: How to go from the phenotype to the genotype. Ann Rev Plant Bio 68: 435-455.
20. Khan MA, Gemenet DC, Villordon A (2016) Root system architecture and abiotic stress tolerance: Current knowledge in root and tuber crops. Frontiers in Plant Sci 7: 1584.
21. Daccord N, Celton JM, Linsmith G, Becker C, Choisne N, et al. (2017) High-quality *de novo* assembly of the apple genome and methylome dynamics of early fruit development. Nat Genet 49: 1099.
22. Velasco R, Zharkikh A, Affourtit J, Dhingra A, Cestaro A, et al. (2010) The genome of the domesticated apple (*Malus*× *domestica* Borkh.). Nat Genet 42: 833.
23. Spangelo L, Fejer S, Leuty S, Granger R (1974) Ottawa 3 clonal apple rootstock. Can J Plant Sci 54: 601-603.
24. Pua E-C, Chong C (1984) Requirement for sorbitol (D-glucitol) as carbon source for *in vitro* propagation of *Malus robusta* No. 5. Can J Bot 62: 1545-1549.
25. Wan Y, Fazio G (2011) Confirmation by QTL Mapping of the *Malus robusta* ('Robusta 5') Derived Powdery Mildew Resistance Gene Pl1. Acta Horticul 903: 95-99.
26. Gardiner SE, Norelli JL, De Silva N, Fazio G, Peil A, et al. (2012) Putative resistance gene markers associated with quantitative trait loci for fire blight resistance in Malus 'Robusta 5'accessions. BMC Geneti 13: 25.
27. Laurent AS, Merwin IA, Fazio G, Thies JE, Brown MG (2010) Rootstock genotype succession influences apple replant disease and root-zone microbial community composition in an orchard soil. Plant and soil 337: 259-272.
28. Zhu Y, Shin S, Mazzola M (2016) Genotype responses of two apple rootstocks to infection by *Pythium ultimum* causing apple replant disease. Can J Plant Patholo 38: 483-491.
29. Weerakoon DMN, Reardon CL, Paulitz TC, Izzo AD, Mazzola M (2012) Long-term suppression of *Pythium abappressorium* induced by Brassica juncea seed meal amendment is biologically mediated. Soil Bio Biochem 51: 44-52.
30. Russo NL, Robinson TL, Fazio G, Aldwinckle HS (2007) Field evaluation of 64 apple rootstocks for orchard performance and fire blight resistance. HortSci 42: 1517-1525.
31. Yang C, Li W, Cao J, Meng F, Yu Y, et al. (2017) Activation of ethylene signaling pathways enhances disease resistance by regulating ROS and phytoalexin production in rice. Plant J 89: 338-353.
32. Zernova OV, Lygin AV, Pawlowski ML, Hill CB, Hartman GL, et al. (2014) Regulation of plant immunity through modulation of phytoalexin synthesis. Mol 19: 7480-7496.
33. Grayer RJ, Kokubun T (2001) Plant–fungal interactions: the search for phytoalexins and other antifungal compounds from higher plants. Phytochem 56: 253-263.
34. Shin S, Zheng P, Fazio G, Mazzola M, Main D, et al. (2016) Transcriptome changes specifically associated with apple (Malus domestica) root defense response during *Pythium ultimum* infection. Physiolo Mol Plant Patho 94: 16-26.
35. Zhu Y, Shao J, Zhou Z, Davis RE (2017) Comparative transcriptome analysis reveals a preformed defense system in apple root of a resistant genotype of G.935 in the absence of pathogen. Int J Plant Geno 8950746.
36. Cobb JN, DeClerck G, Greenberg A, Clark R, McCouch S (2013) Next-generation phenotyping: Requirements and strategies for enhancing our understanding of genotype–phenotype relationships and its relevance to crop improvement. Theore App Gene 126: 867-887.