Optimizing a Protocol for the High-throughput Phenotyping of Armillaria Resistance in Pear

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ABSTRACT

Armillaria root disease is an issue of increasing concern in California that needs to be addressed in a timely manner. The causal fungus, *Armillaria mellea*, is a root pathogen that infects over 300 species of woody plants. Infected trees become more and more stunted over time, losing yields on a gradual basis until the tree eventually dies. The inoculum (partially decayed roots buried under ground) is difficult to eradicate even by fumigation with methyl-bromide, and thus persists in the soil. Economic losses increase as growers shift to higher planting density, which favors disease development. There is no known pear rootstock cultivar resistant to Armillaria, which limits options for replanting infected trees.
Marker-assisted selection has the power of improving breeding efficiency, in comparison to conventional strategies based solely on phenotypic selection, especially for fruit tree crops. In order to identify sources of resistance to be implemented in rootstock-breeding programs, a phenotyping assay for the screening of large numbers of pear accessions is necessary. The objective of this work is to optimize a protocol for high-throughput phenotyping, which we would then use to screen the *Pyrus* core collection (~200 genotypes) of the USDA National Clonal Germplasm Repository in Corvallis, OR. High-quality phenotypic data from such a large and genetically diverse collection could then be used for association studies and eventually develop molecular markers for marker-assisted selection.

OBJECTIVES

Genetic bases of resistance to Armillaria have never been investigated in pear (*Pyrus* spp.), and such studies are just starting in other fruit and nut crops, such as walnut (*Juglans* spp.), peach and almond (*Prunus* spp.). However, although sources of resistance have been discovered within *Prunus* and *Juglans* (Baumgartner et al., 2013; Guillaumin et al., 1991), no sources of resistance are known for *Pyrus*. A broad genetic background needs to be evaluated in order to identify accessions that are tolerant or resistant to Armillaria root rot.

Field and greenhouse-based infection assays have been attempted in other crops (Beckman and Pusey, 2001; Prodorutti et al., 2009), but it takes multiple years for symptoms to be expressed and many plants 'escape' infection. They are therefore not useful for large-scale screening experiments. The use of an optimized in vitro protocol for the high-throughput phenotyping of Armillaria disease in pear will be useful for the rapid and reliable identification of potential sources of resistance, as well as for genome wide association studies. Promising accessions could then be established in the greenhouse or in the field, on infected soil, for confirmation of resistance with a more time-consuming protocol.

Baumgartner et al. (2010) developed an Armillaria in vitro screening assay in grape, and subsequently used a similar approach to identify sources of resistance in rootstocks of walnut (Baumgartner et al., 2013) and almond (Baumgartner et al., 2018). The objective of our work was to:

1. Optimize the Baumgartner protocol for the high-throughput phenotyping of Armillaria resistance in pear.
2. Identify putative sources of resistance to *A. mellea* within the *Pyrus* germplasm.

PROCEDURE

Plant material and micropropagation

A total of twelve pear and one Amelanchier (*Amelanchier* spp.) rootstock genotypes were chosen to test the in vitro screening assay, using genotypes already established in vitro at the Wada Tissue Culture lab at Oregon State University (OSU) and at Sierra Gold Nurseries (SG, Yuba City, CA). These included eight *P. communis*, three *P. betulaefolia*, one *P. spinosa* and one *Amelanchier* spp. accessions (Table 1). Amelanchier clone A15 was bred by Michael Neumüller at Bavarian Centre of Pomology and Fruit Breeding, Hallbergmoos, Germany, and licensed in the USA by Treeconnect.

Table 1: List of genotypes for the first Armillaria inoculation experiment.

Accession #	Inventory #	Taxon	Plant name	Availability	Screening
PI 540929	CPYR_634.001	*P. spinosa*	*P. spinosa*	OSU[1]	Test 2
PI 540945	CPYR_655.001	*P. betulaefolia*	OPR-113	OSU	Test 2
PI 540946	CPYR_656.001	*P. betulaefolia*	OPR-114	OSU	Test 2
PI 540982	CPYR_1379.001	*P. betulaefolia*	OPR-260	OSU	Test 2
--	CPYR_2955.001	*P. communis*	Horner 4	OSU and SG[2]	Test 1 and 3
--	CPYR_2956.001	*P. communis*	Horner 10	OSU	Test 1
--	CPYR_2700.001	*P. communis*	OH-11 (Pyriam)	OSU	Test 1
PI 541415	CPYR_1345.001	*P. communis*	OH×F 87	OSU and SG	Test 1 and 3
PI 541405	CPYR_1572.001	*P. communis*	OH×F 333	OSU	Test 1
PI 541285	CPYR_1164.001	*P. communis*	Winter Nelis	OSU	Test 1
PI 541370	CPYR_726.001	*P. communis*	OH×F 97	SG	Test 3
PI 617679	CPYR_2699.003	*P. communis*	Pyro 2-33	SG	Test 3
--	--	*Amelanchier* spp.	A15	SG	Test 3

[1]OSU: Wada Tissue Culture lab at Oregon State University
[2]SG: Sierra Gold Nurseries (Yuba City, CA)

For micropropagation at OSU, stock shoots were sub-cultured in Magenta GA-7 boxes (Magenta Corp., Chicago, IL, USA) with 40 ml medium per container. The base medium is Pear Rootstock (PRS-propagation) medium, composed of mineral salts (MS) modified to have 2.5× the MS level of mesos (Ca, Mg, P), and with per liter: 2.5 mg thiamine, 250 mg inositol, 30 g sucrose, 4.4 µM N6-benzyladenine (BA), 0.6% agar (A111, PhytoTechnology Labs, Shawnee Mission, KS, USA) adjusted to pH 5.7 and autoclaved for 20 min at 121°C and 15 psi. Shoots were transferred to new medium every four weeks and multiplied to at least 100 shoots for each genotype (total 1,000 shoots). Pear shoot cultures were grown at 25°C under a 16-h photoperiod with an average of 80 µM/m2s irradiance, provided by a combination of cool and warm white fluorescent bulbs. After four weeks, 30 fully grown pear shoots for each genotype were treated with a plant growth regulator (PGR) solution (combination of 5 mM Indole-3-butyric acid (IBA) and 5 mM naphthalene acetic acid (NAA), dissolved in 40% Polyethylene glycol (PEG) 400) to promote root formation. The rooting solution was filter-sterilized (Nalgene Analytical Filterware 150 mL, Thermo Scientific, USA). Shoots were dipped into PGR solution for 2 seconds (method developed by Wada, CPAB 2013 project), then planted on PRS medium with no benzyl adenine (BA) (PRS-rooting medium). Five shoots were planted per Magenta GA-7 box. Treated shoots in containers were covered with aluminum foil for a dark period of one week, and then placed under normal light conditions (same as above). Micropropagation at SG followed a similar protocol, but details are proprietary.
Magenta GA-7 boxes containing the pear shoots were transported to the University of California, Davis (UCD) four weeks after rooting hormone application. All shoots that had developed at least one root were transferred to fresh PRS-rooting medium, one shoot per container, to promote further root growth.

Inoculations and disease assessment

Three subsequent experiments were carried out to test the disease assessment protocol on the 13 genotypes (Table 1): test 1 was carried out on the six *P. communis* accessions from OSU (inoculation date = 11/13/2017), test 2 on the *P. betulaefolia* and *P. spinosa* genotypes (inoculation date = 02/18/2018), and test 3 on the five genotypes received from SG (inoculation date = 05/14/2018). Just before inoculation, plant and root development were assessed by measuring plant height, number of roots longer than 1 cm (“good roots”) and number of roots shorter than 1 cm. One *A. mellea* isolate was used to inoculate different replicates for each genotype. The isolate was recovered from symptomatic pears in Lake County, CA. Inoculum was prepared by homogenizing for 30 s a 7-d culture grown in potato dextrose broth (PDB) with 2.5 mM sodium acetate (25°C, 100 rpm), and then transferring with a sterile 1-ml glass pipette 200 µl of the resulting homogenate (i.e., mycelial fragments) per plant onto the surface of the plant growth medium, as in the protocol developed by Baumgartner in grape (Baumgartner et al., 2010). For each genotype, some replicates were not inoculated (controls). The development of the disease was assessed once per week for up to six or seven weeks. At each weekly assessment, the mycelial growth on the medium surface and the percentage of the plant and of the roots that were necrotic were evaluated; in test 1,

also the percentage of the plant that was chlorotic was measured. Additionally, wilted and dead shoots were noted, and used to calculate percent mortality for each genotype. After the last disease assessment, root tips from one or two inoculated plants per genotype were plated on water agar to confirm infection by recovery of the pathogen in culture. After 10 days of incubation at 25°C, cultures were examined for fungal colonies with the following characteristics: colony diameter of approximately 2 cm, regular colony margin, clampless hyphae embedded in the agar, sparse white aerial hyphae, absence of spores/spore-bearing structures, and the possible presence of immature rhizomorphs (white when embedded in the agar, black when above the surface).

Statistical analysis of phenotypic data

All statistical analyses were performed with R studio (http://www.rstudio.com). At the first three assessments of test 1, categorical measures were used for mycelial growth on the medium surface and root necrosis, thus they were converted to percentages before statistical analysis, to standardize them with the following experiments.
Non-parametric Kruskal-Wallis test was used to assess significant differences among inoculations dates and genotypes for pre-inoculation (plant height, number of total and number of good roots) and post-inoculation measures (mycelial growth on the medium surface, plant necrosis, root necrosis and mortality) at all weekly assessments. Additionally, the Area Under Disease Progress Curve (AUDPC) was calculated for all post-inoculation measures, as indication of the disease development rate, and Kruskal-Wallis test was run as explained above. Correlations between plant height at inoculation with plant necrosis and mortality were evaluated using the Spearman method. Furthermore, the correlation between test 1 and test 3 was evaluated, by calculating the Spearman coefficient between the arithmetic means in Horner 4 and OH×F 87, which were in common between the two tests. The general health status of the plants over time was evaluated by comparing the mortality in the controls versus the cases (inoculated plants) for each genotype at all weekly assessments. Finally, one-way ANOVA was run to determine the ratio of the “between” over “within” genotype variance (F-value) for plant necrosis and mortality (i.e. mortality ~ genotype).

RESULTS AND DISCUSSION

Propagation and rooting success

The number of replicates in acceptable conditions at the time of inoculation was highly variable among genotypes and tests, ranging from five (Winter Nelis, test 1) to 45 (*P. spinosa*, test 2), with a mean of 14 (Figure 1). Test 3 was the most uniform, with approximately 30 inoculated plants per genotype. In general, the majority of the plants in all tests had few, and mostly small roots; however, there were significant differences in rooting (according to Kruskal-Wallis test, $\rho < 0.05$) among inoculation dates and genotypes (Figure 2). Plants inoculated in test 1 showed the largest number of total and good (>1 cm) roots, followed by test 3 and then test 2. However, it is important to note

that plants with no roots were not accounted for in the statistical test. Although all but one of the plants inoculated in test 3 (140) had roots, 12 of the 113 plants inoculated in test 1 had not developed any roots; this, along with the lower number of replicates of some genotypes, may have resulted in an incorrect evaluation of root development in test 1. Significant differences were also observed for plant height, with taller plants in test 3 and smaller in test 2. In test 1, OH×F 87 and Pyriam had the best-developed rooting systems, and OH×F 87 plants were also significantly taller than the other genotypes, while Horner 4 plants were the smallest. In test 2, *P. spinosa* showed higher rooting capacity than the three other rootstocks. Micropropagation of *P. betulaefolia* genotypes was particularly challenging, resulting in only a few small, badly rooted replicates, which contributed to the low scores observed for test 2 in comparison with tests 1 and 3. Among the genotypes inoculated in test 3, Pyro 2-33 showed the more and longest roots, as well as the tallest shoots, whereas A15 had the worse rooting system and Horner 4 the smallest plants, consistently with test 1.

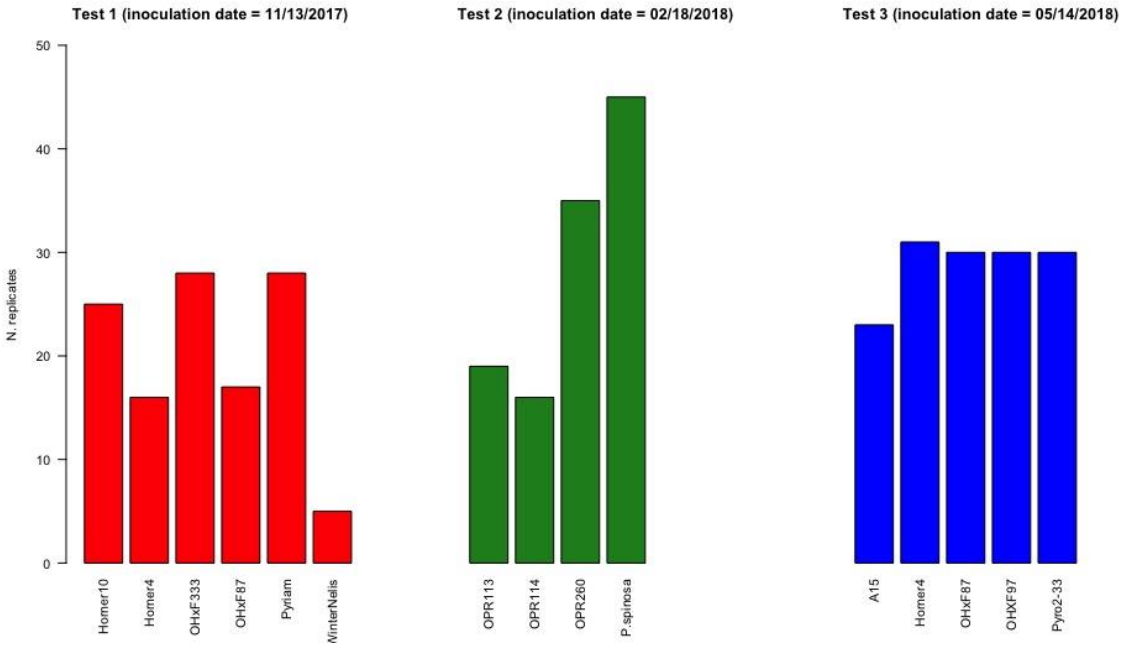

Figure 1: Number of replicates for each genotype at test 1 (red), test 2 (green) and test 3 (blue).

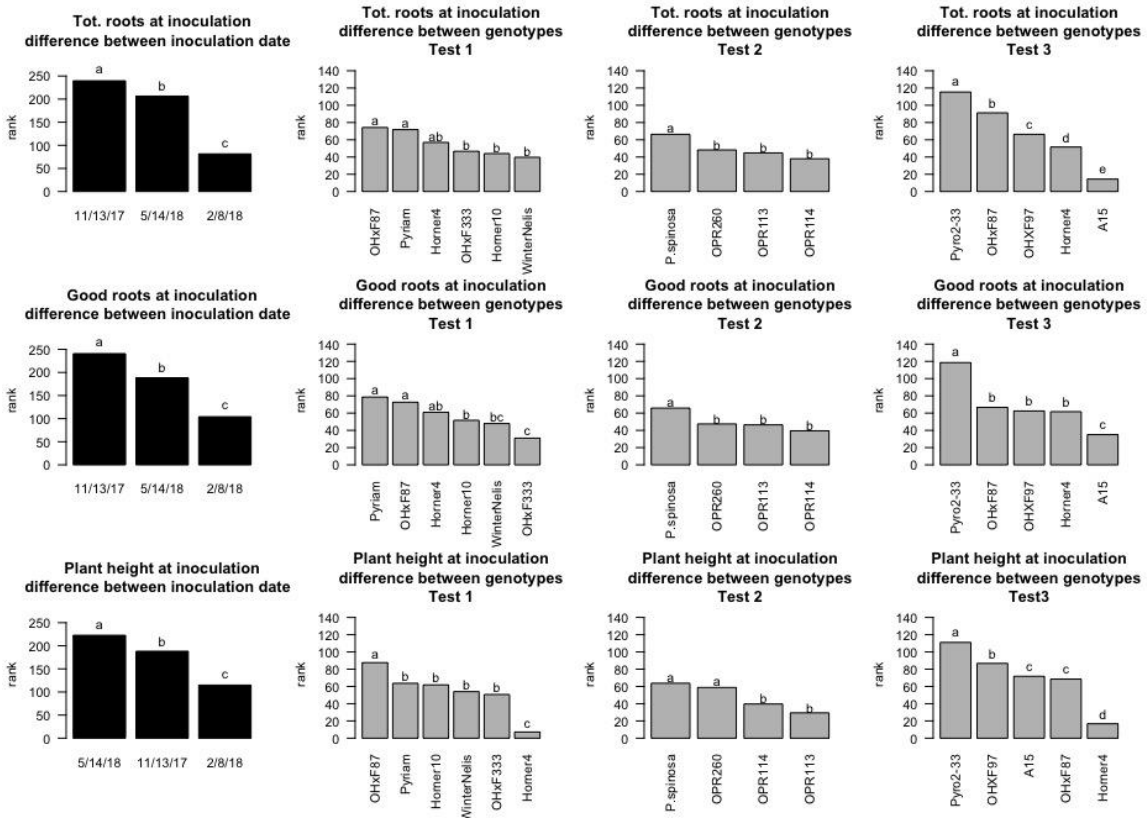

Figure 2: Effect of the inoculation date and the genotype on the total number of roots, the proportion of good (>1 cm) over total roots and the plant height at inoculation. Different letters indicate genotypes that are significantly different from each other according to Kruskal-Wallis non-parametric test ($\rho < 0.05$).

Disease assessments

Disease assessments were carried on until week 7 post-inoculation in test 1; however, all control plants at the last assessment were extremely stunted, showing chlorosis, wilting and in some cases mortality. For the subsequent tests, we therefore decided to stop the disease assessments at week 6, and we did not consider the results of week 7 in test 1. Moreover, plant chlorosis resulted not to be informative of the disease development in test 1, therefore it was not measured in the subsequent tests. Additionally, given the poor quality of most of the plants inoculated at test 2, especially for the three *P. betulaefolia* accessions, we decided not to include the results for this test.

Mycelial growth on the medium surface was very fast for all genotypes in both test 1 and test 3; no growth was observed on non-inoculated controls. Growth was significantly higher and faster (AUDCP) in test 3 than in test 1 (Kruskal-Wallis, $\rho < 0.05$). By focusing on the last assessment and on the AUDPC, we observed significantly less growth in Pyriam than in the other genotypes at test 1, while OH×F 333 had the largest mycelial growth on the medium surface (Figure 3). In test 3, OH×F 87 was the genotype showing less growth, and Horner 4 the one with the largest. Significant

differences were also observed for plant necrosis and mortality (Figure 3). In test 1, Horner 4 appeared the most susceptible genotype, with the highest values of plant necrosis and mortality, while OH×F 87 was significantly more resistant than the other accessions. In test 3, OH×F 87 was confirmed as the most resistant genotype, while Pyro 2-33 appeared as the most susceptible, followed by Horner 4. Despite the larger mycelial growth on the medium surface, plants in test 3 showed significantly less necrosis on the shoots, as well as less mortality. This might be linked to the better status of the plants inoculated in test 3 with respect to test 1. Additionally, there was no correlation between the mycelial growth on the medium surface and plant necrosis and mortality. Root necrosis was inconsistent for each genotype among the different assessments and the AUDPC (Figure 3), which might be because replicates with no, or too small, roots were not accounted for in the Kruskal-Wallis statistics; these were more numerous in test 1 than in test 3. All plated root tips collected from one or two plants per genotype developed *A. mellea* colonies, confirming successful inoculation.

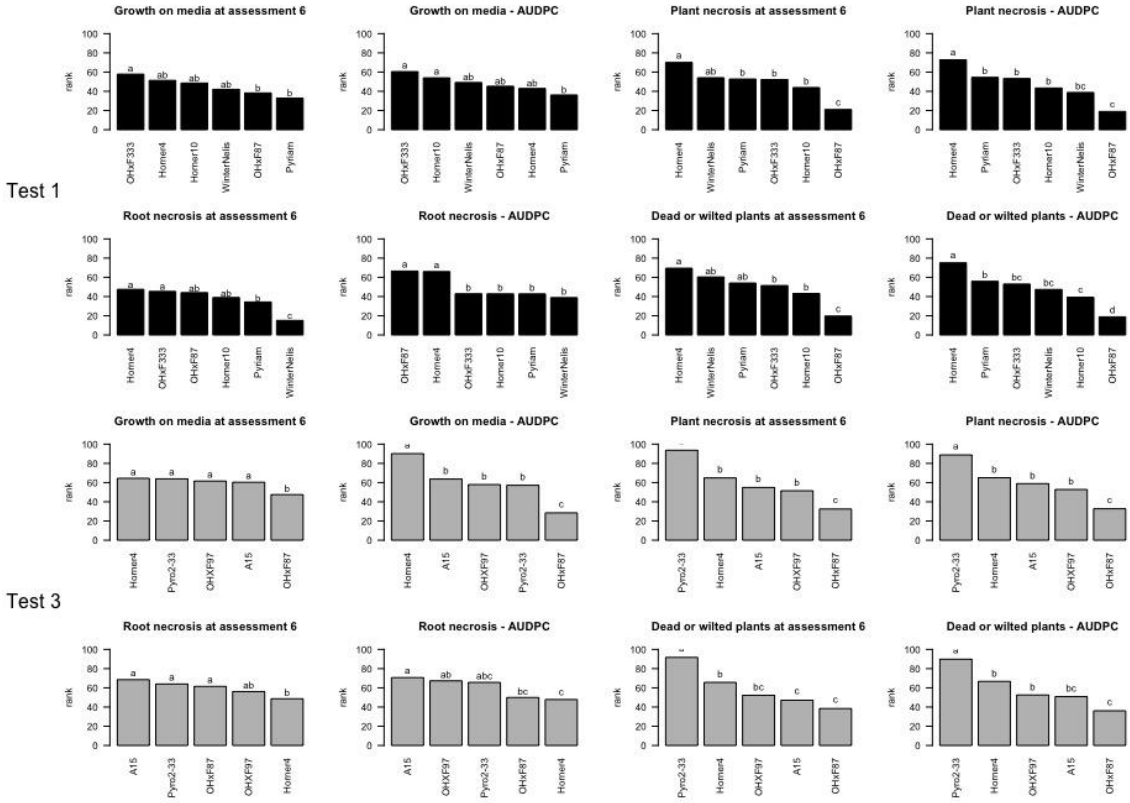

Figure 3: Differences among genotypes at assessments 6 and for AUDPC in Test 1 (black) and in test 3 (grey). Different letters indicate genotypes that are significantly different from each other according to Kruskal-Wallis non-parametric test ($\rho < 0.05$).

All together, our findings suggest that: 1) mycelial growth is a good indicator of successful inoculation, but it does not identify resistant versus susceptible genotypes; 2) root necrosis was too inconsistent to serve as a measure of resistance; 3) poor quality plants, as those inoculated in test 1 with respect to test 3, were generally more

susceptible to the disease. However, a negative correlation was observed between plant height at inoculation and plant necrosis and mortality only in test 1 (Spearman rs = -0.50 and -0.51, respectively, at week 6 post inoculation; Spearman rs = -0.54 and -0.55, respectively, for AUDPC), and not in test 3, suggesting that visual assessment of plant status, more than measurement of plant height and root length, is necessary to determine suitability for inoculation.
When comparing test 1 and test 3 results for plant necrosis and mortality, we observed almost complete correlation for Horner 4, with a Spearman rs = 0.99, while it was lower for OH×F 87 (Spearman rs = 0.80). Therefore, we decided not to analysis the two tests together.

Evaluation of controls

By comparing the number of dead plants per genotype between the controls and the cases, we observed that some of the non-inoculated plants went through increasingly stunting conditions and mortality, in both tests 1 and 3 (Figure 4). This phenomenon was variable among the different genotypes. Horner 4 and OH×F 87 controls were healthy for the entire duration of both experiments. In test 1, some of the Horner 10 controls were dead by week 5, but the majority of the plants were healthy, and the only control plant of Winter Nelis suddenly died at week 6. Controls of OH×F 333 and Pyriam in test 1, and of Pyro 2-33 in test 3 were stunted from the beginning of the assessment, and most of them were dead by week 3 or 4. Finally, in test 3, controls of A15 and OH×F 97 were healthy for the entire duration of the experiment. These observations suggest that some genotypes were less tolerant of the in vitro conditions than others. Even though the root culturing at the end of each experiment confirmed the dead plants to be infected, we could not discern if the high severity observed on certain genotypes was due to their particularly high susceptibility to the pathogen or to their general low tolerance of in vitro conditions.

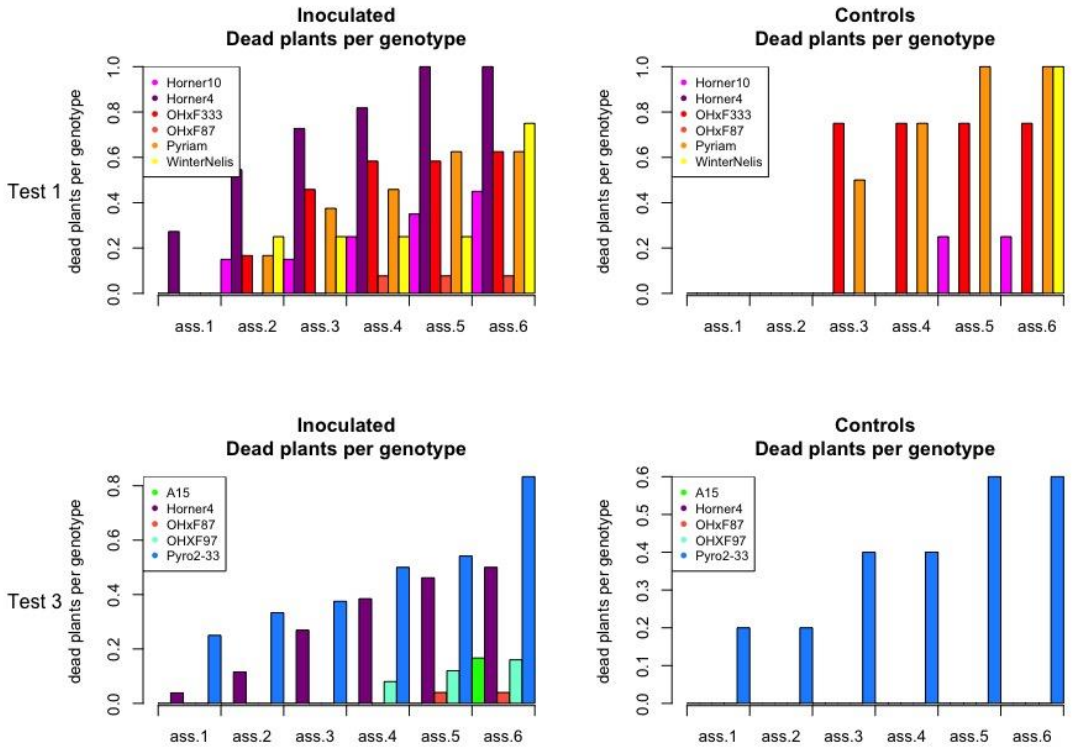

Figure 4: Mean number of dead plants per genotype at each assessment: comparison between inoculated plants and controls at test 1 and test 3.

Variance between and within genotypes

A positive F-value indicates that the variance between genotypes is larger than the variance within genotype. At the sixth assessment (across all tests), the F-value for plant necrosis was 11.12, and for mortality 11.04; considering the AUDPC, the F-value for plant necrosis was 11.99 and for mortality 10.63. In all cases the genotype had a significant effect on the phenotypic trait with a $\rho < 2e^{-16}$.

CONCLUSION

The objective of this study was to evaluate and optimize an in vitro screening assay for the high-throughput phenotyping of Armillaria resistance in pear. In year 1, we have carried out three separate experiments on a total of 12 different pear and one Amelanchier accessions, gradually optimizing the disease assessment method. We were able to observe high variability between the genotypes, and low variability within genotype, suggesting that the measured traits are heritable and that the scoring system is reliable. In conclusion, the optimized phenotyping protocol consists of: 1) visual inspection of plant quality before inoculation and selection of the most uniform, healthy

and well-rooted plants 2) weekly assessments for up to 6 weeks post-inoculation; 3) evaluation of mycelial growth on the medium surface as an indicator of successful inoculation and for identification of contaminants; 4) measurement of percentage of plant necrosis and mortality as the most robust and repeatable quantitative traits; 5) final confirmation of successful inoculation by sampling and plate-culturing of the root tips.

The development of a sufficient number of uniform, well-rooted plants for each genotype represents the main challenge for this test. A minimum number of 20 well-developed and healthy replicates seemed to be necessary, considering that some plants will have to be set aside as controls, and contamination from un-wanted pathogens can occur during the six weeks of disease assessment. In order to achieve this minimum number of 20 replicates at inoculation, a much larger number of plants must be propagated, a number that increases with lower rooting capacity of the genotype. Additionally, different species might require different micropropagation protocols, as we observed in this experiment for *P. betulaefolia* and *P. spinosa*. Furthermore, some genotypes have shown low tolerance to the in vitro culture, and this must be assessed and conditions optimized before starting the phenotyping experiment. However, we have recently been successful in growing and rooting plants for a number of different Pyrus species (Sugae Wada project).

The screening protocol presented in this manuscript will enable the large-scale collection of quantitative and repeatable phenotypic data for Armillaria disease resistance in pear, which is fundamental for genome wide association studies. The application of this assay on the Pyrus core collection (~200 genotypes) of the USDA National Clonal Germplasm Repository in Corvallis, OR will also allow us to identify sources of resistance. Difficult and time-consuming greenhouse or field-based inoculations could then be carried out on a small subset of promising genotypes, to validate their resistance.

In this second year of the project we currently are:

- Micropropagating all the 13 genotypes reported above, with the objective of testing them all at once with the new optimized protocol, using a minimum of 20 replicates per genotype at uniform conditions;
- Optimizing the procedure for microscope observations of infected roots, with the objective of determining differences in amount and location of mycelia between susceptible and resistant accessions;
- Planning a survey in commercial orchards that are infected by Armillaria to evaluate the response in the field of some of the rootstocks screened in this study (in particular OHxF 87, which appeared to be resistant in vitro).

Additionally, we recently secured a CDFA grant for the application of this optimized protocol to the *Pyrus* core collection of the USDA National Clonal Germplasm Repository.

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