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

Sara Montanari, David Neale and Charles Leslie, UC Davis Department of Plant Sciences, Davis, CA
Sugae Wada, Oregon State University, Horticulture Department, Corvallis, OR
Kendra Baumgartner, USDA-ARS, Crops Pathology and Genetics Research Unit, Davis, CA

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 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 develop molecular markers that can be used in marker-assisted selection, genotypic and phenotypic data need to be associated via appropriately designed experiments. With a previous funding cycle, we have designed a high-density SNP marker array and we have completed the genotyping of the entire USDA National Clonal Germplasm Repository of Corvallis, OR. Now we need to collect high-quality phenotypic data for association studies. Currently, the only phenotyping protocol applicable to large-scale evaluations for resistance to Armillaria is an *in vitro* assay developed for grape. We want to test this protocol on a small number of pear rootstock genotypes and evaluate its suitability for high-throughput phenotyping of Armillaria resistance in pear.

OBJECTIVES

Genetic bases of resistance to Armillaria have never been investigated in pear, and such type of studies are just starting in other fruit and nut crops, such as walnut, peach and almond. However, while for these crops sources of resistance have been discovered, either among commercial cultivars or from related wild species, no sources

of resistance are known for pear. A broad genetic background needs to be evaluated in order to identify accessions that are tolerant or resistant to Armillaria.
For year 1 of this project, we are testing an *in vitro* assay for the assessment of Armillaria resistance on a small number of pear rootstock genotypes, with the objective to:

1. optimize the protocol for pear;
2. evaluate different type of disease assessments for suitability to high-throughput phenotyping experiments
3. identify sources of resistance to *A. mellea* within the *Pyrus* germplasm.

The use of an optimized *in vitro* protocol for the assessment of Armillaria disease in pear will be useful for the rapid identification of potential sources of resistance. Promising accessions could then be established in the greenhouse or field on infected soil for confirmation of resistance with a more time-consuming protocol.

PROCEDURE

Propagation and rooting of identified rootstock genotypes

Ten different pear rootstock genotypes were chosen for the first test of the Armillaria root disease assessment protocol, basing on the availability of *in vitro* established material at Wada Tissue Culture lab (OSU): six *P. communis* accessions, three *P. betulifolia* and one *P. spinosa* (Table 1).

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

Species	Plant name	NCGR accession #
P. spinosa	*Pyrus spinosa*	CPYR_634.001
P. betulifolia	OPR-113	CPYR_655.001
P. betulifolia	OPR-114	CPYR_656.001
P. betulifolia	OPR-260	CPYR_1379.001
P. communis	Horner 4	CPYR_2955.001
P. communis	Horner 10	CPYR_2956.001
P. communis	OH-11 Pyriam	CPYR_2700.001
P. communis	OHxF 87	CPYR_1345.001
P. communis	OHxF 333	CPYR_1572.001
P. communis	Winter Nelis	CPYR_1164.001

For micropropagation, stock shoots were subcultured 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/m^2s 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. 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).

Preparation of *in vitro* material for inoculation

60 Magenta GA-7 boxes containing the pear shoots were transported to Leslie Tissue Culture lab at UCD four weeks post rooting hormone application. Ten days later, all shoots that had developed at least one root were transferred to fresh PRS-rooting medium, one shoot per Magenta GA-7 box, to promote further root growth.

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.

Inoculations

One *A. mellea* isolate was used to inoculate the pear genotypes 11 days after transfer into the new media. The isolate was recovered from symptomatic pears in Lake County, CA (Fig. 1 and 2). 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 (Fig. 3), as in the protocol developed by Baumgartner in grape. For each genotype, some replicates were not inoculated, as controls.

Figure 1: Symptoms of Armillaria root disease on pear trees: chlorosis and defoliation (on the left) then progress to complete necrosis and death (on the right)

Figure 2: Collection of Armillaria mellea from infected pear trees

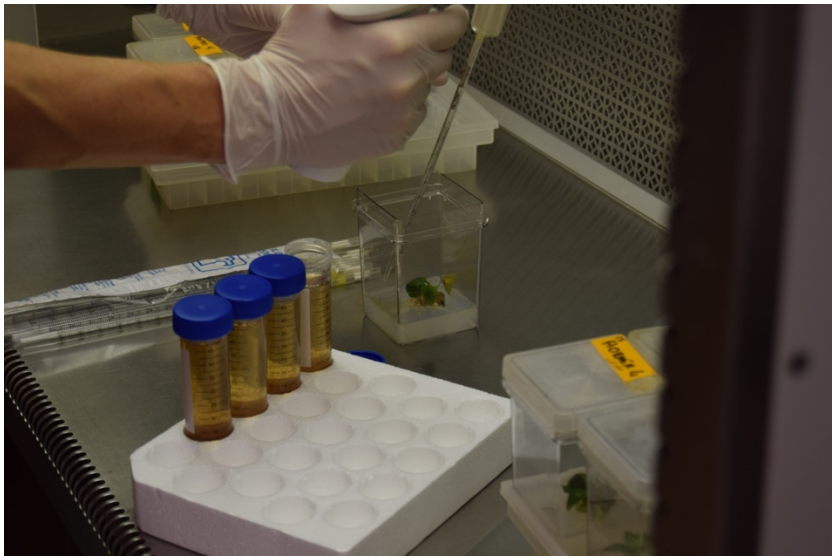

Figure 3: Inoculations of in vitro plants with Armillaria mellea

Disease assessment
The development of the disease was assessed once a week for three weeks (up until now); we estimate to continue disease assessments for another 2-4 weeks. At each weekly assessment, we evaluated the mycelial growth on the medium surface, the percentage of the plant that was necrotic, the percentage of the plant that was chlorotic, the presence/absence of necrosis on roots, and we noted wilted and dead shoots.
We will also collect root tips from dead plants for observations under confocal microscope, with the objective of confirming the presence of *A. mellea* inside the roots.

Additional experiments
We are planning to repeat the entire experiment with a few additional genotypes provided by Sierra Gold Nurseries: *P. commuis* OHxF 97, *P. commuis* OHxF 69, *P. communis* Pyro 2/33 and *Amelanchier*.

RESULTS

Propagation and rooting success

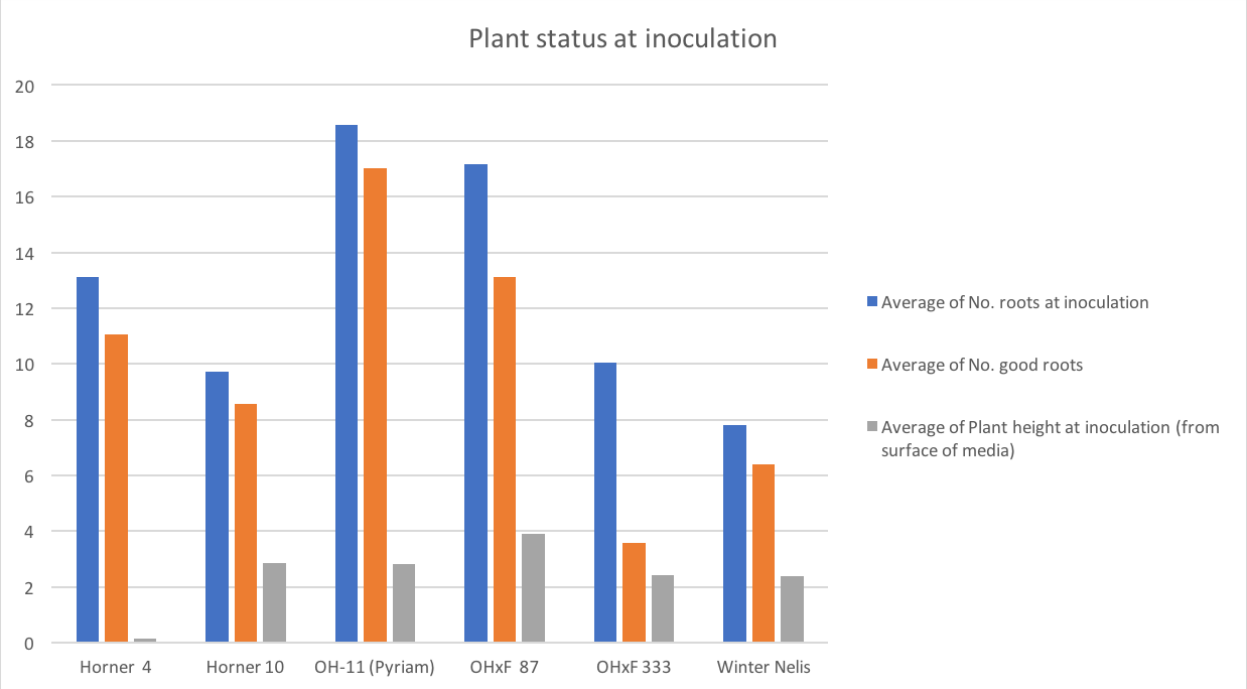

Figure 4: Comparison of average number of roots and plant height at inoculation for the six P. communis genotypes

Plant growth and root development was uneven across the ten genotypes. Both the *P. spinosa* and *P. betulifolia* accessions did not develop any root, therefore they could not be inoculated. Among the *P. communis* accessions, between 5 (Winter Nelis) and 28 (OHxF 333 and OH-11 Pyriam) replicates per genotype were inoculated. At the time of inoculation, OH-11 Pyriam had the better developed root system, while Winter Nelis and

OHxF 333 the worse. Plant height was generally good for all genotypes, with OHxF 87 showing the most vigorous shoots, and with the exception of Horner 4 plants, which grew in a rosette status (Fig. 4 and 5). However, some variability was observed in plant height within genotypes.

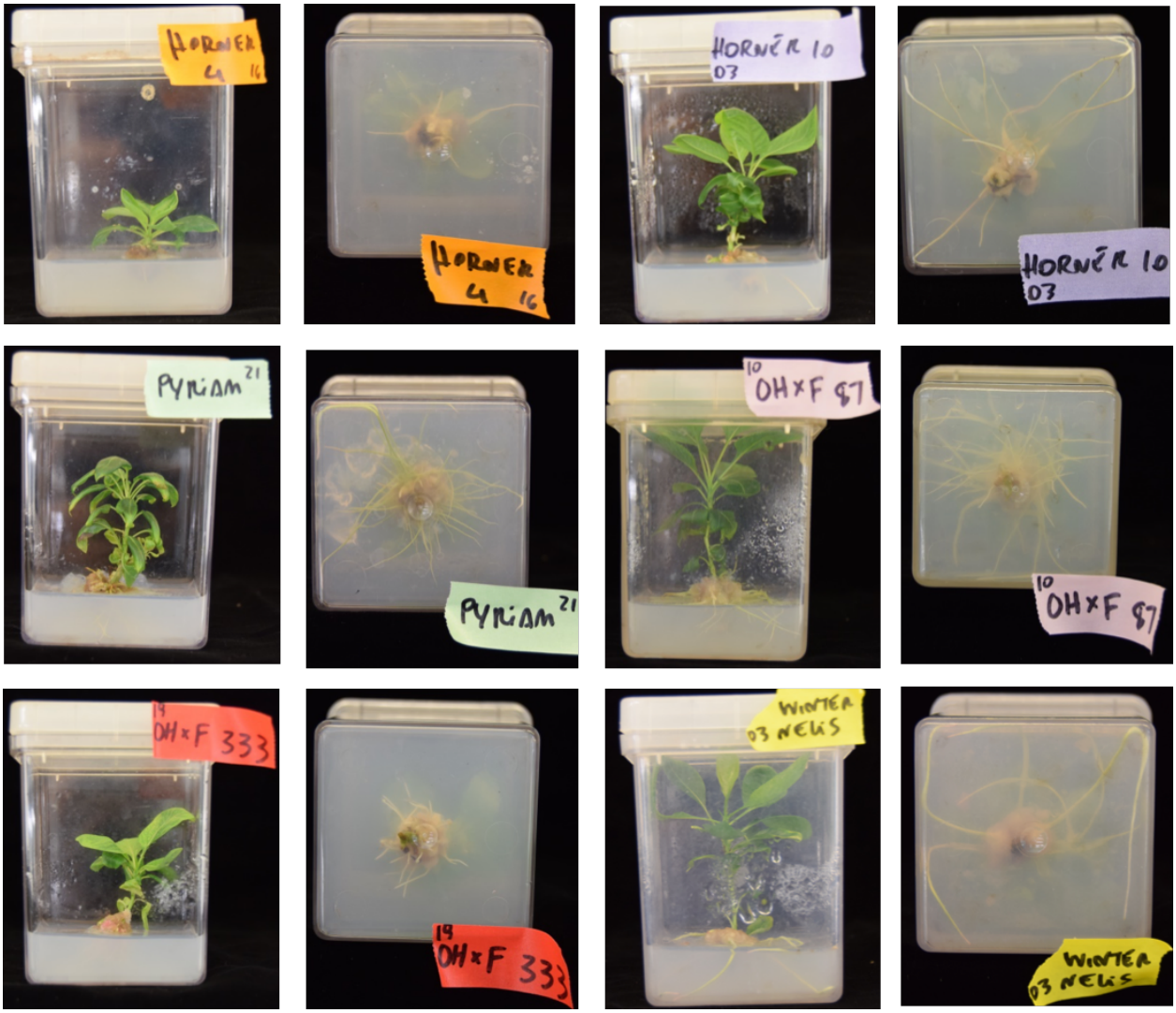

Figure 5: Pictures of one replicate per genotype at the time of inoculation

Disease assessments

Mycelial growth on the medium surface was very fast for all genotypes, with no high variability among replicates; no growth was observed on non-inoculated controls. Visual observations of the shoots showed a clear progress of the symptoms from chlorosis to necrosis and wilting, to mortality in all symptomatic plants (Fig. 6). The disease progress was fastest in Horner 4 (with an average of 73% of the shoot symptomatic by week 3) and slowest in OHxF 87 (only an average of 14% of the shoot symptomatic by week 3) (Fig. 7). Also, by week 3, 71% of the Horner 4 replicates were dead (10 out of 14), while only 7% of the OHxF 87 ones (1 out of 15) (Fig. 8). Severity was low in OH-11 Pyriam and OHxF 333 at week 1, and it progressed towards a medium-high level in week 3

Figure 6: Disease development at week 1 and week 3 on one replicate per genotype

(with respect to the high and low severity observed in Horner 4 and OHxF 87, respectively). Medium-low severity was observed in Horner 10 and Winter Nelis, with not much variation from week 1 to week 2 and 3.

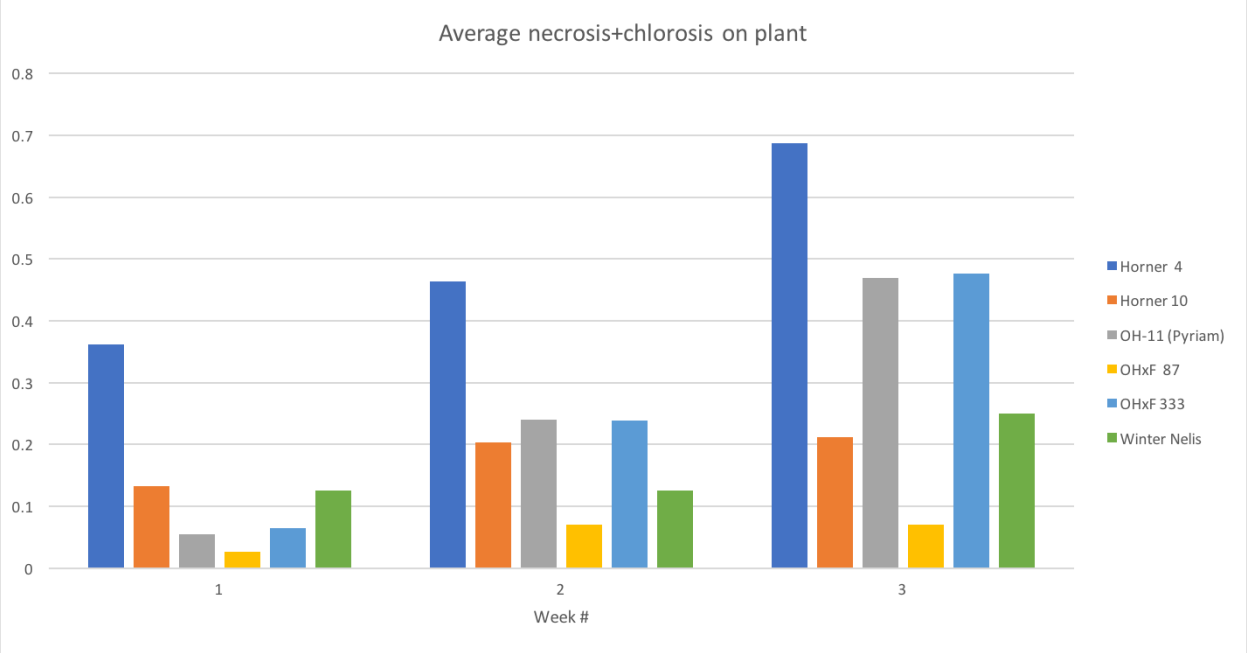

Figure 7: Portion of the shoot that is symptomatic (necrosis + chlorosis) as an average of all the replicates per genotype

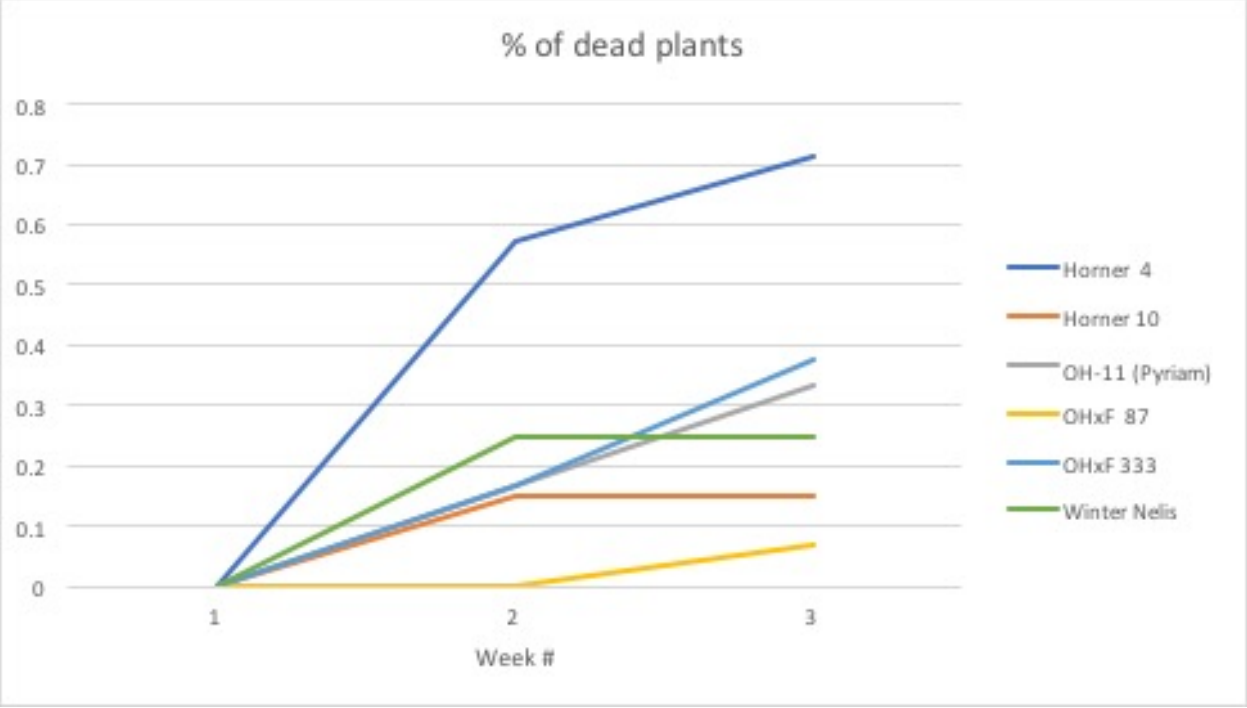

Figure 8: Percentage of dead plants per genotype

Although no mycelial growth was observed on the medium surface of the controls, some shoots appeared stunted. In particular, at week 3 one control from Horner 4, one from Horner 10, one from OH-11 Pyriam and one from OHxF 333 showed chlorosis and necrosis, and two OH-11 Pyriam and three OHxF 333 controls were dead. Microscope observations will help clarify if inoculated plants died because of *A. mellea* infection or deficiencies due to long *in vitro* culture.

We calculated the Pearson correlation coefficient between plant height and number of good roots (longer than 1 cm) from one side, and chlorosis+necrosis and mortality from the other side. While the number of good roots did not appear to affect the status of the plant (r^2 = -0.25 and -0.12, respectively with chlorosis+necrosis and mortality), plant height seemed to have a larger impact (r^2 = -0.48 and -0.46, respectively with chlorosis+necrosis and mortality). More specifically, the entire range of severity and mortality could be observed in smaller plants, while taller shoots were rarely symptomatic. Fig. 9 clearly shows that the portion of plant symptomatic is always lower than 0.2 in plants taller than 4.5 cm, and Fig. 10 that no mortality was observed by week 3 in plant taller than 3.5 cm.

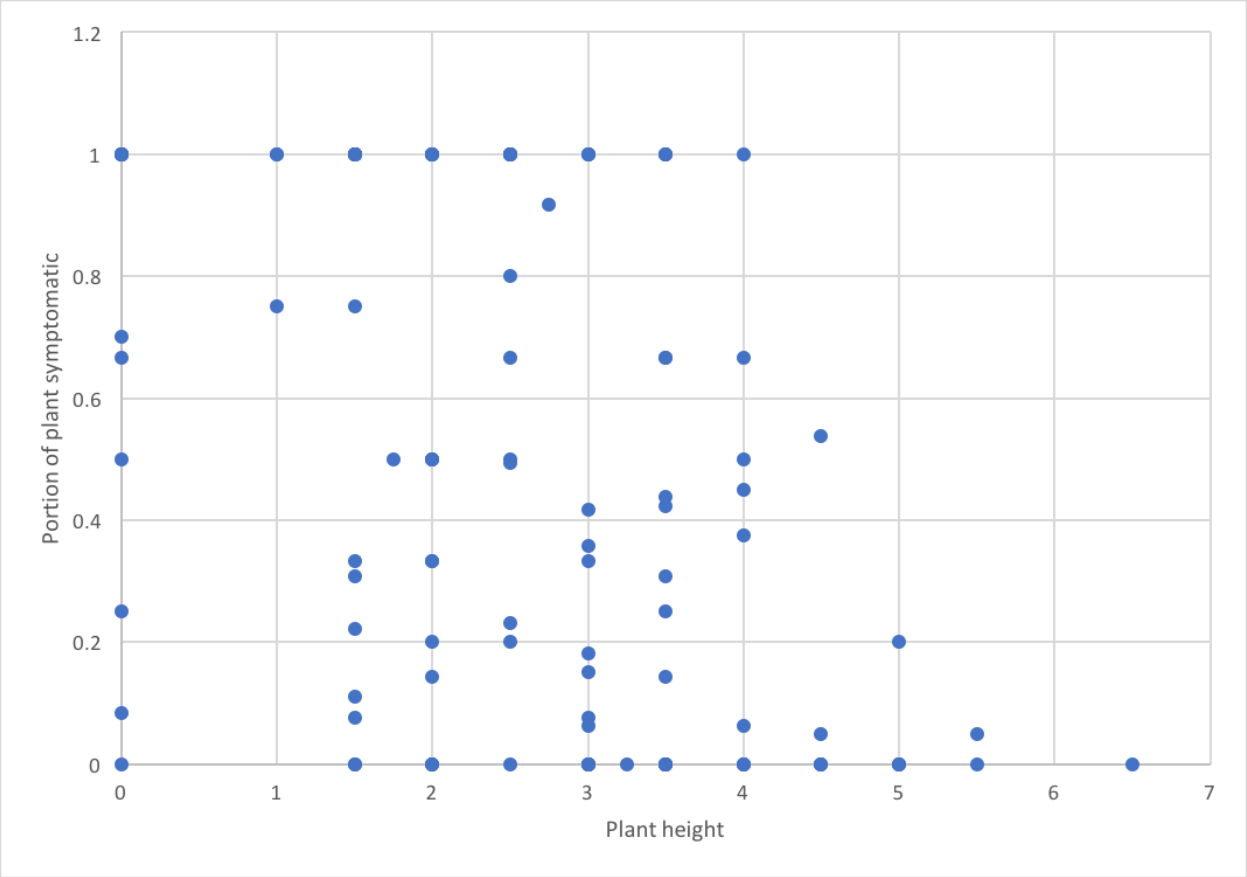

Figure 9: Scatter plot of plant height (in cm) vs portion of the plant symptomatic (chlorosis+necorsis) at week 3

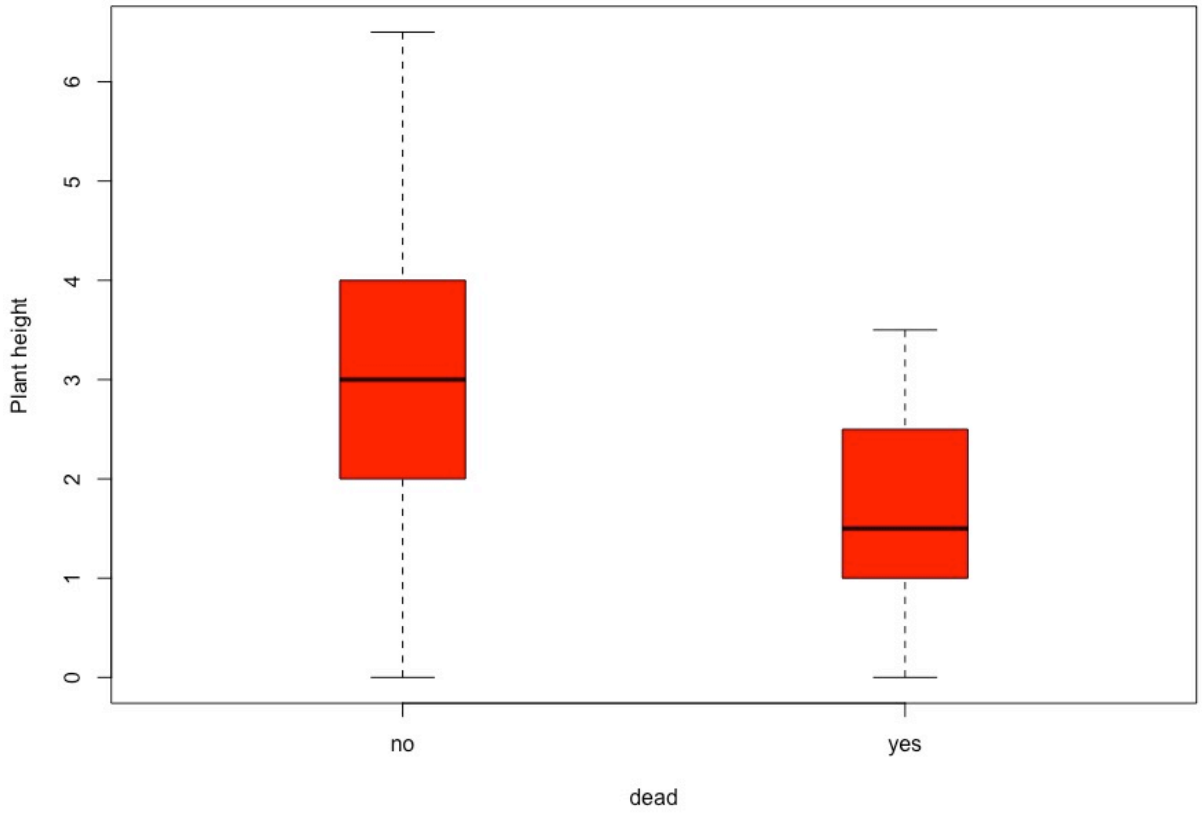

Figure 10: box plot of mortality (plant alive or dead) vs plant height at week 3

DISCUSSION

We started the first experiment for the optimization of a protocol for the high-throughput phenotyping of Armillaria resistance in pear. Even though the disease assessment is still in progress, we were able to identify weaknesses and strengths of the protocols, and start elaborating solutions for its improvement.

The first major challenge regards the *in vitro* plant propagation and rooting of *Pyrus*. Wild species, such as *P. spinosa*, and Asian rootstock species, such as *P. betulifolia*, are interested as potential sources of resistances; however, root growth for those accessions failed in our experiment. Furthermore, while we were more successful with the *P. communis* accessions we tested, plant growth and root ability was uneven across the six genotypes, and shoot height at the time of inoculation appeared to affect the susceptibility of the plants. Therefore, it is imperative to find solutions to improve root ability and *in vitro* growth of both *P. communis* cultivars and other species accessions.

Different characters were measured at the disease assessments, and some of them showed variability across the genotypes (in particular, portion of the plant symptomatic

and number of dead replicates per genotype). Statistical analysis at the end of the experiment will be performed in order to identify the measurements that would be more suitable for high-throughput phenotyping and reliable for association studies.
Despite the small scale of this first experiment and the challenges we encountered, we were able to observe variability in susceptibility among the six inoculated *P. communis* genotypes. Interestingly, OHxF 87 appeared resistant in this first *in vitro* experiment. Unfortunately, no systematic data is available from field observations for this genotype, and further experiments will be fundamental to confirm this resistance. Greenhouse or field inoculations will be especially important for such validation. However, currently a reliable protocol for the Armillaria root disease assessment in greenhouse or filed does not exists.

In conclusion, more experiments need to be carried out to optimize the inoculation protocol for high-throughput phenotyping of Armillaria resistance in pear. However, the preliminary results here presented are promising.