**Phytoplasma Influence on Michigan Carrots**
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The overall goal of this project is to determine the degree to which phytoplasma or virus infections contribute to woodiness in Michigan carrots.

Woody carrots pose choking hazards to babies and thus can be used only in pureed forms of baby food and not as diced carrots. To produce carrots appropriate for diced baby food forms, it is important to identify causes of woodiness in carrots and to be able to monitor and manage these factors in the field. Woodiness typically is associated with carrot bolting and can be caused by numerous drivers including environmental conditions (e.g., drought) and potentially pathogens.

Previous Malmstrom lab investigation of an initial set of aster yellows phytoplasma (AYp) genotypes identified three phylogenetic groups in Michigan vegetable fields: One group, associated with strains known to cause "witches' broom" symptoms, had a phylogeographic distribution suggesting that it originates from local Michigan sources. A second group, associated with strains found to cause "bolt" symptoms, appeared to be circulating within a broader geographic area. The third group was less common and less well characterized. In Michigan, it is thought that the 'witches' broom' phenotype may not contribute much to the problem of carrot woodiness. Grower experience suggests that the foliage of plants infected with this genotype typically dies before harvest, so that these carrots do not generally enter the processing stream. Thus, the "bolt"-associated AYp genotypes may be of particular interest for monitoring and control.

We therefore are examining two questions: (1) **To what degree is carrot woodiness associated with bolting?** In other words, **are there any non-bolting carrots that are woody?** and (2) **Is woodiness associated with phytoplasma or viral infection?**

In 2018, we addressed these questions by first monitoring field infections associated with early bronzing of carrot leaves (Fig. 1) and witches' broom (Fig. 2). In early August, we identified carrots with these symptoms in the field, tagged them, and followed them over time. As expected, most of these carrots 'melted' away before harvest or developed weak foliage that would prevent the roots from being harvested.

**Figure 1. Bronzed foliage in field-grown carrots**

**Figure 2. Witches' broom (WB) symptoms**

Next we examined carrots exhibiting late-season bolting, bronzing, and witches' broom (WB). To reduce the numbers of woody carrots harvested, growers for Gerber are asked to rogue blooming (white-flowered) carrots from fields prior to harvest. However, carrots that become woody without bolting and those that are woody and just beginning to bolt but not flowering, may be missed in this process and contaminate the harvest. We thus targeted carrots at bolting stages before display of white flowers, which might be missed in rogueing.

In late August – September 2018, we harvested and rated 524 carrots from four fields producing carrots for Gerber (Figs. 3–5). To facilitate comparisons, we harvested 189 non-bolting carrots, 72 non-bolting with witches' broom, 259 bolters, and 4 bolters with witches' broom. The bolting carrots were rated by stage as follows: Stem evident (S); bud evident (B); green flower (G); emerging white flower (E); white flower (W). The majority of bolters selected were at the stem, bud, or green flower stage, as these are the stages most likely to be overlooked in rogueing and thus of most concern.

Figure 3. Ellen Cole (MSU) and Drew Afton (Nestle/Gerber) evaluate carrots for sampling.

Figure 4. Drew Afton helps transport samples to the vehicle.


Figure 5. Carrots were chilled on ice and transported quickly to MSU for processing.

In the lab, every carrot was washed (Fig. 6), photographed, and then rated for hairiness, knobbiness, number of laterals, and hardness.

Figure 6. MSU Senior Garrett Mulanix processes carrots in the Malmstrom Lab.

Gerber did not have a standard measure of carrot woodiness that could be used, so we devised a metric based upon how hard a root was to cut under standardized conditions (Fig. 7). We then evaluated relationships between carrot traits and field phenotypes (e.g., bolting stages).


Figure 7. Hardness and phenotype of carrot roots along standardized scale of cutting difficulty from 1 (easy) to 5 (very hard) and 6 (very very hard—not shown).

For analysis of DNA (phytoplasma) and RNA (viruses), we took samples of roots, stems, and foliage from all samples and froze them in our freezers (Fig. 8).


Figure 8. Carrot tissue samples prepared for DNA and RNA analysis

Next, we tested methods of nucleic acid extraction to determine the best approach for detecting phytoplasma and viruses in these carrots. The extraction stage is determinative of downstream results but often given only cursory attention. We took care with this stage because we know from previous work that it is all too easy to miss signal if extractions are not optimized. In the case of carrots, there are some specific challenges. The root tissue can be tough (particularly if woody) and it is full of pectin, which co-precipitates with DNA and can confound analyses. The stems and foliage contain inhibitors which, if not removed, can inhibit downstream tests and cause false negatives. We conducted a series of tests that convinced us that working with foliage and stems is the best choice and we modified our homogenization processes to make this most feasible. For example, carrot foliage when homogenized tends to create foam, which needed to be reduced.

We next deep-sequenced the DNA of one set of carrot stem and root tissues with AYp infection and assembled longer-length AYp contigs. To get broader understanding of the distribution of AYp genotypes, we further Sanger-sequenced AYp sequences from numerous leafhoppers and symptomatic carrots.  We assembled contigs compared the identity of the sequences, and created phylogenetic trees to evaluate their relationships.

# Results so far

In our estimation, carrots with the greatest toughness ratings (5-6) are likely to be problematic in processing lines, and those rated hard (4) may also be sub-ideal. We found that carrots at the green flower stage (G) or higher were more likely to be hard (rated 4-6). However, hard (4) and very hard carrot roots (5-6) were found in all flowering stages, including those with zero signs of bolting (N), not even a beginning stem (Fig. 9). Thus, the answer to question 1-- **are**

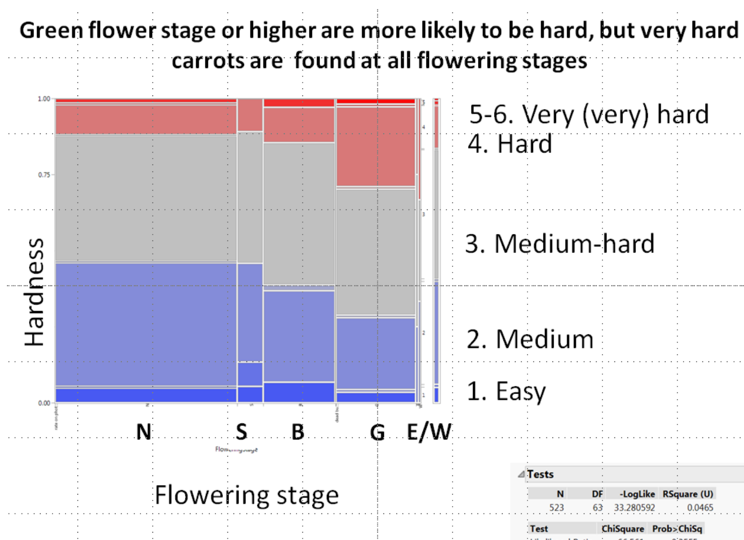


Figure 9. Numbers of carrots with hardness ratings 1-6 as a function of flowering stage: N-not flowering; S-stem; B-bud; G-green flower; E-emerging white flower; W-white flower)

**there any non-bolting carrots that are woody?**—is, yes, there are. This means that rogueing white-flowering carrots will reduce but not eliminate the harvest of woody carrots.

Next we considered carrots with AYp witches' broom (WB) symptoms. As expected, carrots with these symptoms are significantly hairier, with substantially greater numbers rated as H2 (maximally hairy, blue color in Fig. 10).

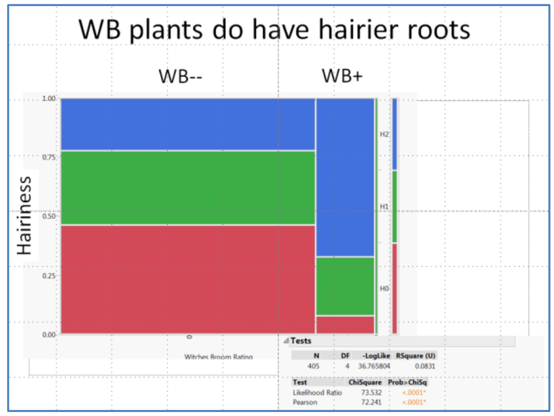


**Figure 10. Distribution of root hairiness ratings (H0-H2) in carrots with and without witches' broom (WB).**

However, carrots with witches' broom symptoms are not harder to cut than carrots without these symptoms (Fig. 11). We did not find any WB-carrots that exhibited the highest hardness ratings (5-6). Thus, it appears that WB symptoms alone are not an indicator of increased woodiness. With regard to question 2-- **Is woodiness associated with phytoplasma or viral infection**?—we conclude that later-season infection that causes WB in non-bolting carrots is likely not contributing significantly to the woodiness problem in the carrot cultivars and environment examined. We thus turn our attention to infections related to bolting and other potential causes of woodiness.

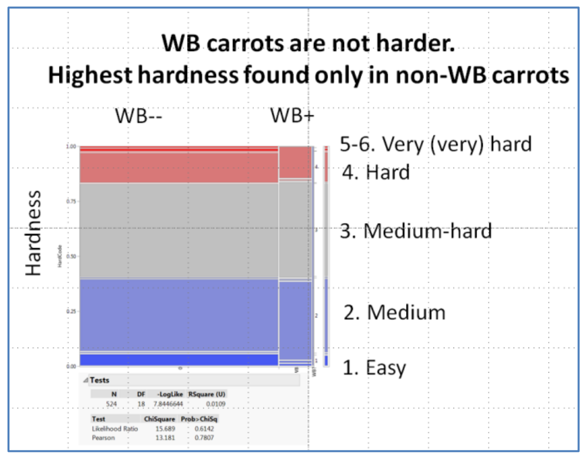


**Figure 11. Distribution of hardness scores in WB- and WB+ carrots.**

Some of the woodiness problem may be related to carrot germplasm. The carrots that earned the toughest ratings included thin, poorly colored carrots with roots that were entirely woody (Fig. 12). It is possible that this phenotype results from introgression of weedy genotypes into the seed mix or field population of seedlings.

**Figure 12. Extremely woody carrots.**

We also examined other phenotypes with substantial root hardness, including roots with a tough white central layer and ones with significant knobs (Fig. 13). The cross-section on the right in Figure 13 shows an emerging knob, which is much harder than the surrounding tissue.

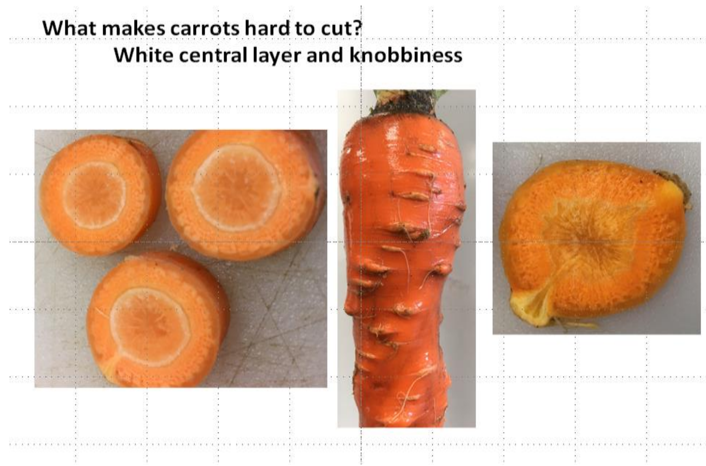


Figure 13. Carrots with these phenotypes also earned high hardness ratings.

Knob formation on roots has potential to be contributing significantly to carrot woodiness. Roots with the greatest amount of knobs (rating K2) were more likely to be hard (4) or very hard (5-6) than those with lesser ratings (Fig. 14).

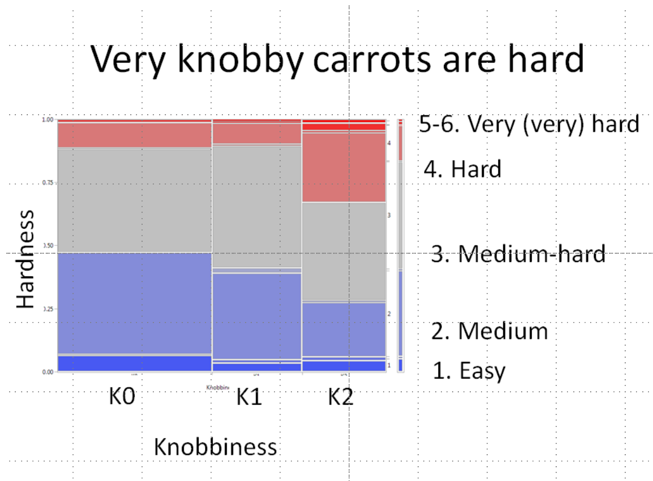


Figure 14. Distribution of hardness ratings by knobbiness class. K2 is maximal knobbiness.

We found that there was a significant association between knobbiness and bolting. Bolting carrots (stem to white flower stages) were more likely to be substantially knobby (K2) than either normal carrots or those with witches' broom (WB) symptoms (Fig. 15).

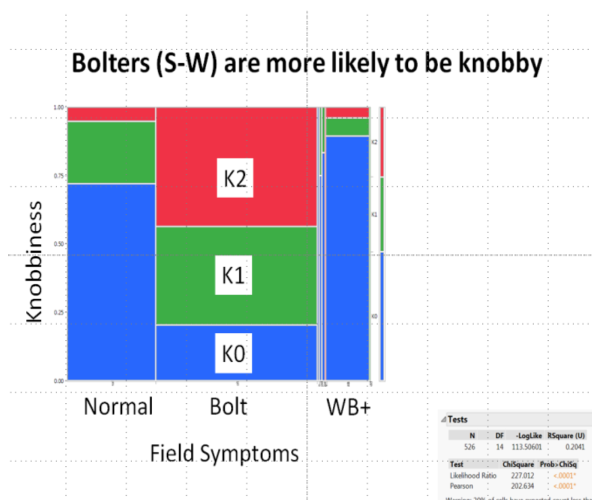


Figure 15. Knobbiness classes as a function of field symptoms.

Moreover, knobbiness increases with flowering stage (Fig. 16). Normal (N) carrot roots include few individuals with the top knobbiness rating (K2). The percentage with K2 ratings increases precipitously in association with stem emergence and enlarges further through the B, G, and E/W stages. Thus, rogueing of carrots with emerging white flowers (E) or white flowers (W) will reduce the harvest of very knobby carrots, but will not remove the significant numbers of very knobby carrots in earlier bolting stages, or the few found in non-bolting carrots. Critical next questions include the degree to which these knobs can pass into the baby food processing stream and the extent to which they are linked with infection.

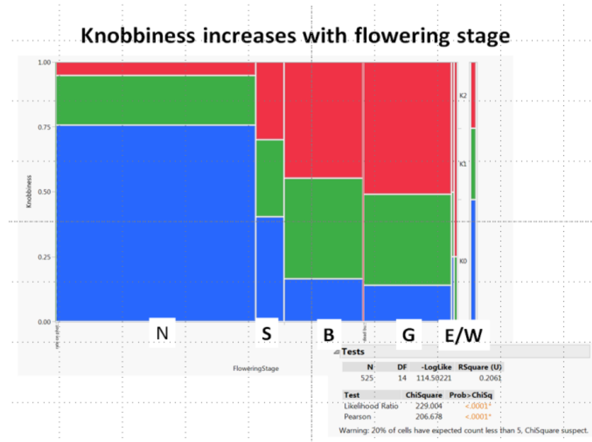


**Figure 16. Knobbiness classes as a function of flowering stage. K2 (red) is the maximum rating.**

In our DNA analyses, we have sequenced 16S-23S intergenic spacer regions from a suite of phytoplasmas collected from carrots and leafhoppers and then looked at their genetic relationships with phylogenetic trees. This expanded set of sequences has revealed more detail. There are at least three groups of AYp evident including one similar to other witches' broom types, one similar to onion yellows and bolt-causing strains, and an intermediate type (Fig. 17). The first two types seem to have about equal representation in Michigan carrot and celery fields.

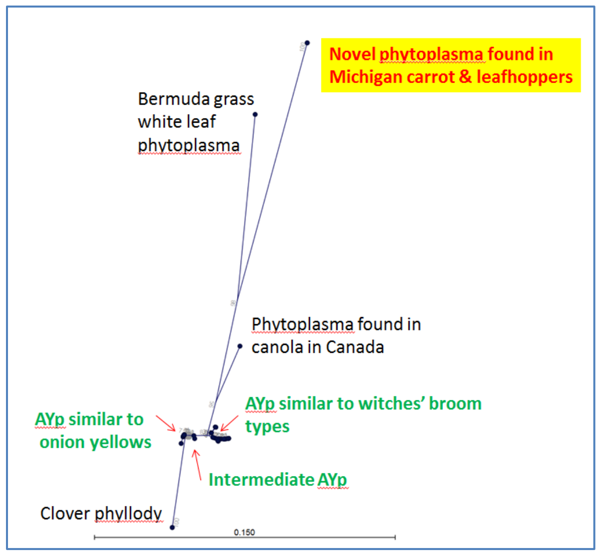


**Figure 17. Simplified neighbor-joining tree of DNA sequences from 16S-23S intergenic spacer regions from Michigan phytoplasma and others.**

The most striking development is that we found a novel never-before-seen phytoplasma in both carrot and leafhopper in two counties, suggesting that it is circulating within the region. Genetically, it groups with phytoplasma found in canola in Canada and with another called "Bermuda grass white leaf" that infects grasses, but it is still quite distinct, as shown by the long branch length in the figure. This finding raises several key questions. For example, how pathogenic is this new phytoplasma? Is it moving from canola or sugar beets, or even grasses, into carrots? Does it pose any threat to carrots?

Next steps in investigating the extent to which woodiness is associated with phytoplasma or viral infection include completion of DNA testing, additional sequencing including deep-sequencing of the carrot virome, and in-depth analysis of relationships between carrot phenotypes and detected phytoplasma/virus infections as well as possible interactions with nematodes and/or Pythium.