Canadian Agri-Science Cluster for Horticulture 2

Progress Report April 2016

Activity 15 Potato 14

Development of a Rapid and Sensitive Triplex Nested Real-time PCR Method for Quantification of Verticillium in Soil

Name of Lead Researcher:

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Names of Collaborators and Institutions:

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Activity Objectives (as per approved workplan):

This activity is aimed at developing a fast and accurate method for the quantification of *V. dahliae* (pathogen of potato and other Canadian crops such as tomato, alfalfa and sunflower) and other *Verticillium* species of importance in Canadian potato soils. A Real Time PCR method will be developed for other *Verticillium* species possibly in Canadian soils that are pathogens or produce microsclerotia to interfere with the reliability of traditional plate counting methods. Towards the end of the project, the method will be adapted to a commercial laboratory setting to provide fast enough turn-around of analyses that producers can decide on control options prior to planting their crop.

Research Progress to Date (use plain language):

Based on the literature review and discussion with Dr. Guillaume Bilodeau (CFIA, Ottawa), the *Verticillium* species chosen to examine by real time PCR in potato soils were species producing microsclerotia (*V. dahliae*, *V. tricorpus*, *V. longisporum*, *V. isaacii*, *V. klebahnii*, and *Gibellulopsis nigrescens* (formerly *V. nigrescens*)) and the non-microsclerotial plant pathogenic species *V. albo-atrum*. A collection of these species was generated from existing isolates in the Soil Ecology Laboratory and the laboratory of Dr. Fouad Daayff at the University of Manitoba, the Canadian Fungal Culture Collection of AAFC, Dr. Katherine Dobinson of AAFC, and Dr. Guillaume Bilodeau of CFIA. An isolate of *V. longisporum* was obtained from Dr. Krishna Subbarao of UC Davis originally isolated from cauliflower in California. The Soil Ecology Laboratory had to import the isolate with CFIA certification and the laboratory required development of protocols and isolation means to obtain CFIA PP1 certification to work with this species. We also received an isolate of *V. longisporum* from AAFC derived from canola in Manitoba.

Care was taken to develop pure cultures from each isolate The pure cultures were then screened for morphological traits and IGS sequencing to confirm species identification. A method for extraction of genomic DNA from dried potato soils in Manitoba was optimized by examination of various commercial extraction kits, varying weight of soil and time of disruption using a bead beater, and determination of genomic DNA yield and quality by spectrophotometry.

Real time primer sets for the *Verticillium* species were obtained from the literature, the laboratory of Dr. Guillaume Bilodeau or designed internally from IGS sequencing and the Genebank database. Genomic DNA from pure cultures of the *Verticillium* species were screened using the primers and dilution series of the DNA extracts and at least one primer set selected specific to a species used in subsequent studies. Next the selected primers where screened against genomic DNA of the species added to soil. For *V. dahliae*, varying amounts of microsclerotia (5, 10, 25, 50, 100, 150, 250 microsclerotia g^{-1} dry soil) were added to soil and for all other species varying amounts of genomic DNA from pure culture extracts.

Soil from 20 commercial potato fields in Manitoba were examined. These were plated onto Sorensen's medium and 62 colonies producing microsclerotia were randomly selected and turned into pure culture isolates for further primer screening. The isolates were identified by morphological and IGS sequencing to be *V. dahliae* (44), *V. tricorpus* (16), *V. klebahnii* (1) and *G. nigrescens* (1). The species real time primer sets were verified also against these isolates.

Soils from multiple locations within select commercial fields in Manitoba were analyzed for *Verticillium* counts by traditional wet plating and also screened using the species real time primers. The results validated the random culture selection the *V. dahliae* and *V. tricorpus* were the dominate species. Further, plate counts of *Verticillium* were clearly affected by the presence of both species. Thus the counts are not specific to *V. dahliae* which is considered a serious pathogen of potato whereas the economic importance of *V. tricorpus* is uncertain.

Soil and plant samples from trials on 6 commercial potato fields in NB and PEI were obtained from Dr. Bernie Zebarth. The samples were used to validate the protocols developed for Manitoba. The soils and plant sections were plated for microsclerotial forming *Verticillium* as well real time PCR analysis for *V. dahliae*, *V. tricorpous* and *V. longisporum*, as well as conventional PCR for *V. albo-atrum*. Low plate counts were found. There was a low frequency and amount of *V. dahliae*. The soils were expected to have high *Verticillium*, especially *V. albo-atrum*. We thus screened the primer set for *V. albo-atrum* more carefully and found it to not be consistent. Thus we developed a new primer set for *V. albo-atrum* which is consistant for *V. albo-atrum*.

Our next find was CT values of real time assays for clay soils from MB and the Maritims were very high. This can be either from low levels of *Verticillium* DNA or inhibition from organic matter in clay soils. Further, on sand soils the real time assays could quantify down to about 20 microsclerotia per gram of soil. This is not good enough in our opinion to provide a robust commercial method to base management decisions on. We are now evaluating a sucrose flotation-centrifugation of 100 g of soil to extract DNA from more soil and limit inhibitors from soil.

Lastly, the next innovation was to improve the calibration of the real time assays. We initially used with great success DNA from isolates in culture. However, the method was not very consistent. We thus developed cloned calibration genes unique for each species to use in real time assays moving forward.

We have begun working with the PSI laboratory in MB to train them in sample processing, extraction and analysis of *Verticillium* species. That work is being funded through other projects.

Extension Activities (presentations to growers, articles, poster presentations, etc.):
Verticillium and fumigation. M. Tenuta. McCain Producer Meeting. Portage La Prairie. October 22, 2015.
UofM Potato Projects Under the Growing Forward 2 Program. M. Tenuta. Keystone Potato Growers Spring Production Meeting. Portage La Prairie, MB. February 22, 2016.

Early Outcomes (if any) or Challenges:
We are making excellent progress in developing real time PCR assays for the Verticillium species in potato soils and plants. The methods have recently been improved by using cloned genes for calibration and extracting from 100g than just 0.4 g of soil. This is a theoretical increase of 250 times the concentration of Verticillium DNA extracted and should reduce sample to sample variation because of greater soil mass extracted.

Key Message(s):
There are several species of *Verticillium* in potato soils. One species in MB in particular, *V. tricorpus*, produces microsclerotia and inflates *Verticillium* plate counts. The real time PCR assays developed here overcome this problem. We found V. tricorpus to not be pathogenic to Russet Burbank. This is good for growers in that they likely do not have to control it, but it means conventional plating soil testing is useless for Manitoba.

The Agri-Science Cluster for Horticulture 2 is generously funded by nearly 50 industry partners and Agriculture and Agri-Food Canada's AgriInnovation Program, a Growing Forward 2 initiative.