

2003 O.P.V.G. EXECUTIVE SUMMARY REPORT

PROJECT TITLE: ECOLOGICAL/EPIDEMIOLOGICAL STUDIES OF THE HIGHLY VIRULENT GROUP C AND D FORMS OF THE BACTERIAL SPOT PATHOGEN

RESEARCHER: Dr. Diane A. Cuppels, Agriculture and Agri-Food Canada, London

PROJECT OBJECTIVE: To characterize Ontario strains of the highly virulent groups C and D of the bacterial spot pathogen (first discovered in Ontario fields in 2000) and to develop the tools (including a real-time fluorogenic PCR-based assay for quantifying bacteria on greenhouse plug seedlings) needed to perform ecological and epidemiological studies of them.

METHODOLOGY:

Growth of bacterial spot Groups A, B, C and D on plug seedlings and on field tomato and pepper plants. Five-week-old tomato (cv. N1082) and pepper seedlings (cv. Super Hungarian Hot), propagated in an environmentally-controlled plant growth chamber, were sprayed (10^7 bacteria/ml) with representative strains of the four bacterial spot groups: DC97P6B (Grp A), DC93-1 (Grp. B), DC00T15A (Grp C) and DC00T7A (Grp D). Ten-gram samples of leaves were collected at 0, 1, 3, 6 and 8 days after inoculation and the bacterial populations were determined. The experiment was done in triplicate. In our field study, we had four tomato (cv. H9553) and four pepper (cv. Super Hungarian Hot) plots; two of the tomato and two of the pepper plots were sprayed with Kocide every 7 days starting one week after planting and ending eight weeks after planting; the tomato plants also were sprayed with Bravo 500 F. We inoculated the plants with the four bacterial spot strains 10 days after planting and followed the bacterial populations for 50 days. Fruit lesion number and yield per plant were determined 13 weeks after planting.

DNA extraction. DNA was extracted from bacterial cultures by a freeze-boil method (crude extract) or by Qiagen Genomic-tips (purified DNA). DNA also was extracted and purified from the wash water of tomato leaves that had been sprayed with various concentrations of the bacterial spot pathogen. The negative control consisted of wash water from leaves that had not been exposed to bacteria. Each sample was done in triplicate.

Real-time quantitative PCR. The Roche LightCycler Probe Design Software was used to select bacterial spot primers for use in the Roche LightCycler Instrument. Each PCR was performed in a 20- μ l volume (1.6 μ l of 3 mM $MgCl_2$, 1 μ l primer mix, 2 μ l SYBR Green I mix, 14.4 μ l water and 1 μ l DNA template) in a capillary tube. The amplification program began with 10 min at 95 C followed by 37 cycles (ramp rate of 20 C/sec) of 95 C for 1 sec, 60 C (annealing temperature) for 10 sec and 72 C for 12 sec. Standard curves were prepared using kit-purified genomic DNA from the bacterial spot strain DC93-1 or from leaf wash water containing various concentrations of bacteria. The threshold cycle, C_T , is the cycle number at which fluorescence emission crosses an arbitrarily defined threshold within the logarithmic increase phase. By comparing a sample's C_T value with that found on a standard curve of C_T vs. log concentration, one can determine the amount of cells or DNA in that sample.

RESULTS:

Characterization of Group C and D strains of bacterial spot. There are four known groups of the bacterial spot pathogen: A, B, C and D. We have isolated 112 Ontario strains of the bacterial spot pathogen since 2000 and the majority belong to Group D. In previous years, Group B was the dominant group. Fortunately, only one strain, a Group C, was resistant to copper. When sprayed on tomato plants grown in a plant growth chamber, the four groups grew at equivalent rates with the group D strain reaching a slightly higher population level. When sprayed on pepper plants, the Group A and D strains grew better than the

Group C strains (as expected since this group does not cause disease on pepper) and, surprisingly, the B strains. None of the strains reached the populations levels that were observed on the tomato plants. On field tomato plants, Groups B, C and D grew as well as they did on the tomato plants in the plant growth chamber; the Kocide-Bravo sprays reduced their numbers by 90-97%. The Group A strain did not do as well on the field tomato plants and its populations were sharply reduced by the sprays. Group A, originally isolated from pepper, grew better on field pepper plants than on chamber-grown pepper plants. Group D also grew well on field pepper plants but Group B did not. On field pepper leaves, the Kocide sprays sharply reduced the populations of all four groups. The Kocide-Bravo sprays also significantly reduced the number of fruit lesions per tomato plant for Groups A, B and D. The majority of the lesions found on tomato fruit were formed by Group B or D strains, even though the Group C strain grew as aggressively as the Group B and D strains on leaves. The majority of the lesions found on Group A-sprayed plants were formed by Group B and D strains. Differences in total tomato yield for the various treatments were not statistically significant. However, Kocide-Bravo treatment did increase the yield of disease-free fruit.

Real-time quantitative PCR. PCR primers are short strands of DNA that are required to initiate DNA replication by the Taq DNA polymerase in a polymerase chain reaction (PCR). The primers we had previously chosen for quantitative PCR in the Roche LightCycler, BSX7/8, gave a good correlation between bacterial number and fluorescence intensity but the presence of primer-dimers reduced the sensitivity of the assay significantly, particularly when used with plant samples (4000 bacteria/PCR reaction). To increase the sensitivity and accuracy of the assay and to ensure that the best possible primers were being used in the assay, we employed the Roche LightCycler Probe design software to select new primer sets. We needed to select primers that also could be used with fluorogenic hybridization probes. We have tested several sets of primers-hybridization probes with both crude bacterial extracts and purified bacterial DNA. We found that the best primer set was BSX9/10; it gave a single band (290-bp) of the correct size with no primer-dimer bands. The detection level using spiked leaf wash water samples, where the DNA had been extracted and purified using the Epicentre kit, was approximately 5 to 10 bacteria/PCR reaction, or 500 to 1000 bacteria per 10-g leaf sample. We plan to test this BSX9/10-based quantitative PCR assay with leaf samples from the plug greenhouses in the spring of 2004.

SUMMARY AND CONCLUSIONS

The majority of the 112 bacterial spot strains isolated from Ontario fields over the past three years belong to Group D. Only one of these strains, a Group C, is copper-resistant. When we compared representative strains from each group, only Group D grew aggressively on both tomato and pepper plants (both chamber-grown and field-grown). The Group A strain, originally from pepper, did not grow as well on field tomato plants as it did on chamber-grown tomato plants. The Group C strain grew aggressively on chamber-grown and field-grown tomato plants but did not form as many lesions on fruit as did the Group B and D strains. Group C strains are not pathogenic for pepper. Kocide sprays significantly reduced the growth of all four groups on tomato and pepper leaves. They also reduced but did not eliminate the number of lesions formed on tomato fruit. The yield of disease-free fruit was higher for Kocide-Bravo sprayed plants. In this experiment, the lesions on pepper fruit were very rare and thus we did not record their numbers.

We have improved our real-time quantitative PCR assay for determining the bacterial spot populations present of greenhouse-grown tomato and pepper seedlings by designing a set of primers-hybridization probes that not only has a good amplification efficiency and also allows us to detect 5 to 10 bacteria/PCR reaction. With this level of sensitivity, we should be able to find as few as 250-500 bacteria per 10-g sample of symptomless plug plant leaves.