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White Paper

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Bacteriophage: A Viable Bacteria Control Solution

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Abstract

An increasing number of bacterial plant pathogens have been reported to be tolerant or resistant to bactericides in present-day use. Bacteriophages (bacterial viruses) were evaluated as alternative control agents using bacterial wilt and bacterial spot of tomato as test systems. It was found that 92% of three-week old tomato plants pretreated 4 hours (h), 1 day (d), 3 and 5 d with bacteriophages (phages) before inoculating with *Ralstonia solanacearum* remained healthy, whereas 95% of untreated plants had some manifestation of disease. Similarly, 91% of three-week old tomato plants given phage specific for *Xanthomonas perforans* 4 h and 1 d before exposure to the pathogen had less than 12% defoliation. In contrast, 80% of untreated plants displayed more than 12% disease severity. Although all plants were pretreated with phage by pouring 50 ml around the base of plants, after eleven d phage was isolated from all terminal leaflets from a phage-treated bacterial spot infected plant. Higher numbers of phage were obtained from the youngest leaflet as well as from a blossom on the 21st d. Plants treated with phage both in the greenhouse and field demonstrated less spread and incidence of bacterial spot than those given water or copper.

INTRODUCTION

Bacterial plant diseases affecting important agricultural crops can result in considerable damage and serious economic loss worldwide. They are becoming more difficult to control because bactericides in present-day use are not as effective as they have been in the past. Development of copper-tolerant and copper-resistant strains has caused major difficulty in controlling bacterial plant pathogens with copper compounds. Tolerance and resistance to copper has been reported for many bacterial pathogens affecting important crops such as cherry (Sudin et al., 1989, 1993), ornamentals (Scheck and Pscheidt, 1998), pear (Loper et al., 1991), pepper (Marco and Stall, 1983; Martin et al., 2004; Ritchie and Dittapongpitch, 1991), tomato, (Jones et al., 1991a, 1991b), and deciduous woody plants (Scheck et al., 1996) to name only a few. The isolation of

bacterial pathogens resistant to other agrochemicals, for example streptomycin-resistant strains from apple (Burr et al., 1988; Chiou and Jones, 1991; Jones et al., 1991), cherry (Sudin and Bender, 1993), pear (Loper et al., 1991), pepper (Ritchie and Dittapongpitch, 1991), tomato (Stall and Thayer, 1962), and woody plants (Scheck et al., 1996) further emphasizes a steady evolving serious agricultural problem.

As a result, there is a critical present-day need to identify other effective bactericides for bacterial plant pathogens. This has been exemplified by studies directing toward evaluating different chemical agents for control of bacterial ring rot of potato (Secur et al., 1988), bacterial speck of tomato (Colin and Chafik, 1986), bacterial spot of tomato (Jones and Jones, 1983), citrus canker (McGuire, 1988), and halo blight and bacterial brown spot in beans (Schwartz and McMillan, 1989).

Shortly after their discovery as bacterial lytic agents, bacteriophages (bacterial viruses) were investigated not only for control of human diseases (Stent, 1963), but also for control of plant diseases (Okabe and Goto, 1963). Although bacteriophages (phages) were exploited successfully in past years to control bacterial plant diseases (Thomas, 1935), they were abandoned due to emergence of bacterial mutants resistant to the phages employed (Katznelson, 1937). Until recently plant pathologists have generally rejected the use of phages because there was no way to circumvent the appearance of phage-resistant mutants.

A new technology was introduced (Jackson, 1989), which unlike the common past use of only one phage for prevention of disease utilizes a mixture of three to eight different phages including h- (host-range) phages (Balogh et al., 2003; Flaherty et al., 2000). H-phages are spontaneously derived from their wild-type parent phages, and have been found to lyse not only their parent wild-type bacteria but also phage-resistant mutants originating from their parent bacteria. They were named host-range mutants because of their capacity for attacking this extended range or number of hosts. If a bacterial control mixture is composed of phages including h-phages, any phage-resistant mutants arising in a bacterial-pathogen population will be destroyed by heterologous phages in the multiphage composition.

Experiments were designed to evaluate the feasibility of utilizing phage mixtures as a viable alternative for controlling bacterial plant diseases using bacterial wilt and bacterial spot of tomato as test systems.

MATERIALS AND METHODS

Isolation, characterization, production and preparation of AgriPhage (phage formula manufactured by OmniLytics, Inc.) mixtures were carried out by Dr. Lee E. Jackson. The greenhouse and field trials were conducted at the University of Florida Gulf Coast Research and Education Center in Bradenton under direction of Dr. Jeffrey B. Jones.

Bacterial strains.

Four strains of *Ralstonia solanacearum* and a copper-sensitive strain of *Xanthomonas perforans* (refer to paper presented by Dr. Jeffrey B. Jones at this symposium) were stored in 30% glycerol at -80°C . All bacteria were revived by growth in 0.8% nutrient broth on a rotary shaker at 28°C . Purity of bacterial cultures was checked by streak plating upon nutrient agar. Suspensions were made from overnight

nutrient agar growth by transferring pure colony isolates to 0.01M MgSO₄ ·7H₂O, and concentrations adjusted as indicated by measuring optical density at 600 nm.

Bacteriophages.

Clear-plaque producing phages specific for each host were isolated originally from tomato plant parts, i.e., leaves, stems, etc., soil, or field run-off, stream and river waters. One h-phage for each host was also isolated. Six phages for each host were produced by inoculating log phase cultures [approximately 1 x 10⁸ colony-forming units (cfu)/ml in nutrient broth/0.2% yeast extract/0.25% glucose] with a multiplicity of infection between 0.01-1.0. Vigorous shaking of viral-host mixtures continued for a minimum of 9 h to overnight at 28°C. Bacterial debris and survivors were removed by centrifugation at 10,000 x g for 10 min. Each phage suspension was titered to obtain at least 10⁹ plaque-forming units (pfu)/ml. Appropriate mixtures were prepared and sterilized by passage through a 0.2 μm microbiological filter.

RESULTS AND DISCUSSION

Three-week old tomato plants were pretreated with an AgriPhage mixture specific for *R. solanacearum* by pouring 50 ml around the base of each plant. The plants were pretreated with the phage mixture 4 h, 1, 3 and 5 d before inoculation with a mixture of four different strains of *R. solanacearum*. The phage mixture had a final titer of 10⁸ pfu/ml and the bacterial suspension was 10⁷ cfu/ml. After three weeks, each plant was rated for disease severity (refer to Table I).

Of 53 plants pretreated with phage, 49 or 92% remained healthy. Pretreatment with phage at 4 h, 1, 3 or 5 d appeared to be equally effective in preventing bacterial wilt. In contrast, nineteen of twenty untreated plants or 95% had some sign of disease whether they were slightly wilted (5 or 25%), general wilted (7 or 35%) or dead (7 or 35%).

In a separate study for control of bacterial spot, three-week old tomato plants were pretreated with 50 ml of an AgriPhage mixture of three phages for *Xanthomonas perforans*. Treatment consisted of pouring the phage mixture onto soil around the base of each plant. The titer of the phage mixture was 10⁸ pfu/ml. 4 h or 1 d later, both phage-treated as well as plants not treated with phage were sprayed with 10⁸ cfu/ml suspension of *X. perforans*. All plants were enclosed in clear polyethylene bags, placed in a growth chamber at 28° C for 36 h. The plants were then removed from the bags and transferred to a greenhouse. Seven days after inoculation with *X. perforans*, disease incidence was higher on those plants that did not receive the phage pretreatment than those that did. Two weeks after inoculation with the pathogen, the plants were rated for bacterial spot severity (see Table II). It was found that 91% (29 of 32 plants) of phage-treated plants had less than 12% defoliation, whereas 80% of untreated plants had greater than 12% defoliation.

On the eleventh day after pathogen inoculation, terminal leaflets were aseptically retrieved from all leaves of an AgriPhage-treated plant and assayed for presence of phage. Phages were discovered in all leaflets analyzed with the highest number of phages appearing in the youngest leaflet (Figure 1). On the 21st day, one blossom was sampled and found to contain 429 pfu/mg (Figure 1). The translocation of phage confirms Boyd et al. (1971) wherein they detected phage in stem and root tissue two

weeks after immersing one-month old tomato plants in a phage lysate specific for *Agrobacterium tumefaciens*.

Experiments were set up to determine if an AgriPhage mixture sprayed onto tomato seedlings in the greenhouse will curtail manifestation of bacterial spot. Seedlings in styrofoam planter flats, each consisting of 128 cells for growth of one seedling per cell, were sprayed either with well water, copper as Kocide, or an AgriPhage mixture. Prior to spraying, a centrally-located seedling within each flat was inoculated, by needle-infiltration of a true leaf, with a 0.01M MgSO₄ •H₂O suspension of a copper-sensitive strain of *X. perforans* prepared from overnight growth on nutrient agar. The bacterial inoculum was equivalent to 3 x 10⁸ cfu/ml. After inoculation, spray treatments continued on a daily basis throughout an eighteen-day period, after which the number of seedlings displaying bacterial spot lesions were tabulated. As displayed in Figure 2, of those seedlings receiving water, 55 (43%) and 41 (32%) showed bacterial spot lesions, whereas in the flats treated with AgriPhage only 4 (3%) and 2 (2%) were infected (Figure 3). The number within each cell indicates number of lesions per plant, and the circled cell signifies the original *X. perforans*-inoculated seedling. Flats sprayed with copper had 27 (21%) and 28 (22%) infected seedlings (Figure 4).

Tomato transplants treated in the greenhouse as described above (see column one, Table III), were transferred to the field for further treatments (column two, Table III). Disease ratings for each treatment were recorded in columns labeled I, II, III, and IV with an average for the four replicates (Table III).

In all treatments when AgriPhage was included in the application, the average disease ratings were lower [water/phage (2.25), copper/phage (2.75), phage/water (3.5), and phage/phage (2.5)], with the exception when phage treatment in the greenhouse was followed by copper in the field [phage/copper (5.5)]. Disease incidence in watered plots was lower than those treated with copper [water/water (4.75), copper/water (4.25), and phage/water (3.5)]. In treatments when copper was used in the field, the average disease ratings were higher [water/copper (5.75), copper/copper (5), and phage/copper (5.5)], than water and phage treatments.

CONCLUSIONS

Results from these experiments indicate the potential of phage mixtures as effective preventives both in the greenhouse and field for control of bacterial plant pathogens and their diseases. Some benefits associated with the use of phage mixtures for control of bacterial plant diseases are phages are highly specific, nontoxic, biodegradable, and amplify or increase in number upon infection of their hosts.

Unlike most agrochemicals, phage exhibit narrow specificity of action, killing only targeted, pathogenic bacteria. Phages will not attack other bacteria, many of which are beneficial to plants and soil ecosystems. Inasmuch as phages are specific, they will not hurt agricultural workers, consumers, wildlife including night crawlers and beneficial insects such as bees, domestic animals, plants, as well as soil macro- and micro- fauna and flora. Humans have been exposed for decades to phages in water (Bergh et al., 1989), and will consume phages when they eat raw produce (Jacobsen, 1936; Kennedy et al., 1986), cheese (Deane et al., 1953; Whitehead and Cox, 1936), delicatessen meats (Kennedy et al., 1984), fresh oysters (Denis, 1975; Whitehead and Cox, 1936), raw milk (Whitman and Marshall 1971), and other foods such as fermented products

If a chemical preparation is too dilute, it will be ineffective in its control of pathogenic bacteria. If too concentrated it will be not only toxic to workers, but also to plants undergoing treatment. If a mixture of phages is more dilute than intended, more phage offspring will be produced within infected hosts. If a more concentrated phage mixture is applied, it will neither harm growers nor plants, but merely supply more phages than required to kill targeted bacterial pathogens.

Inasmuch as phages are composed of protein and nucleic acid, eventually they will be broken down by proteases and nucleases secreted by various species of soil bacteria and fungi. Amino acids, nucleotides, and other end products of microbial hydrolase activity will be absorbed as nutrients by soil inhabitants including plants. Unlike many agrochemicals such as copper, phages do not persist in the environment. Phage compositions offer a control alternative for bacterial plant pathogens with no biological risk or environmental pollution.

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Tables

Table 1. Exposure of tomato plants to *Ralstonia solanacearum* after pretreatment with AgriPhage.

Plants evaluated on 21st day after exposure to wilt pathogen									
Experiment Number	Time between phage treatment and exposure to wilt pathogen	Total Number of Plants	Healthy Plants		Number of Diseased Plants				
			Number	Percent	Slightly Wilted	General Wilting	Dead	Total	Percent
1	4 hours	10	10	100%	0	0	0	0	0%
1	3 days	10	8	80%	0	0	2	2	20%
1	5 days	10	10	100%	0	0	0	0	0%
1	Sub-total Treated	30	28	93%	0	0	2	2	7%
1	No Treatment	10	0	0%	2	2	6	10	100%
2	4 hours	10	8	80%	0	2	0	2	20%
2	1 day	13	13	100%	0	0	0	0	0%
2	Sub-total Treated	23	21	91%	0	2	0	2	9%
2	No Treatment	10	1	10%	3	5	1	9	90%
Summary of Tomato Bacterial Wilt Experiments 1 and 2									
1,2	Treated Plants	53	49	92%	0	2	2	4	8

Table 2. Exposure of tomato plants to *Xanthomonas perforans* after pretreatment with AgriPhage.

Experiment Number	Time between phage treatment and exposure to spot pathogen	Total Number of Plants	Number of plants of given disease severity (Disease severity in % defoliation)									
			0-3	3-6	6-12	Total	%	12-25	25-50	50-75	Total	%
1	4 hours	9	2	4	3	9	100%	0	0	NA	0	0%
1	No Phage Treatment	10	0	1	3	4	40%	2	4	NA	6	60%
2	4 hours	10	1	4	4	9	90%	1	0	0	1	10%
2	1day	13	1	4	6	11	85%	1	0	1	2	15%
2	Sub-total Treated	23	2	8	10	20	87%	2	0	1	3	13%
2	No Phage Treatment	10	0	0	0	0	0%	1	7	2	10	100%
Summary of Tomato Bacterial Spot Experiments 1 and 2												
1,2	Treated Plants	32	4	12	13	29	91%	2	0	1	3	9%

Table 3. AgriPhage and Kocide field treatments of tomato transplants that had undergone similar treatments in the greenhouse.

Greenhouse	Field	I	II	III	IV	Avg.
Water	Water	4	4	5	6	4.75
Water	Copper	6	6	6	5	5.75
Water	Phage	3	2	2	2	2.25
Copper	Water	4	5	5	3	4.25
Copper	Copper	5	6	4	5	5
Copper	Phage	3	4	2	2	2.75
Phage	Water	4	4	2	4	3.5
Phage	Copper	6	6	4	6	5.5
Phage	Phage	2	2	4	2	2.5

Key: Disease severity in percent of defoliation:

1 = 0% 2 = 0-3% 3 = 3-6% 4 = 6-12% 5 = 12-25% 6 = 25-50%

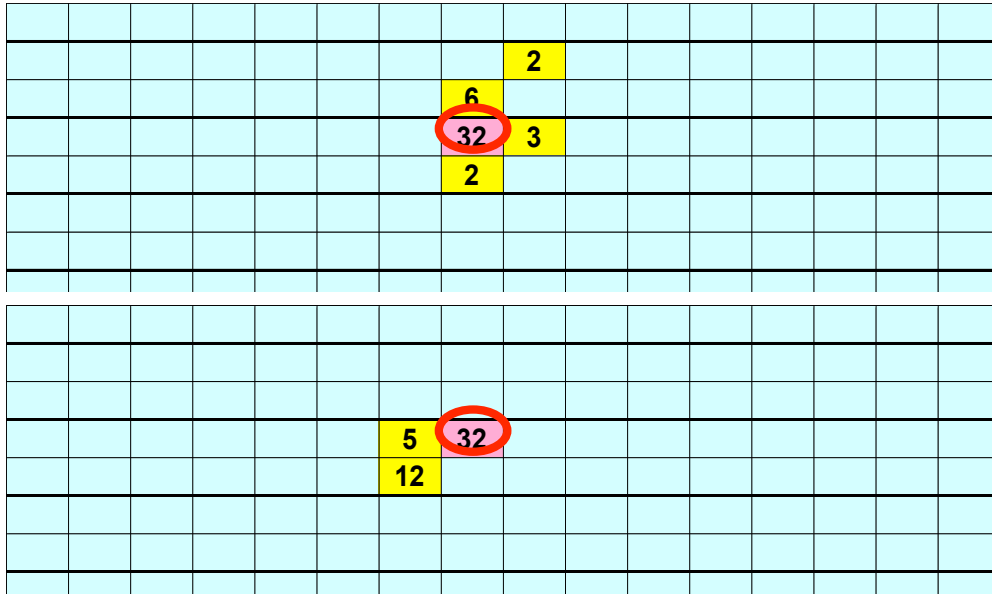


Fig. 3. Spread and incidence of bacterial spot lesions in tomato seedlings in two planter flats after infecting a centrally-located seedling (circled) with *Xanthomonas perforans*. Seedlings were sprayed daily with AgriPhage.

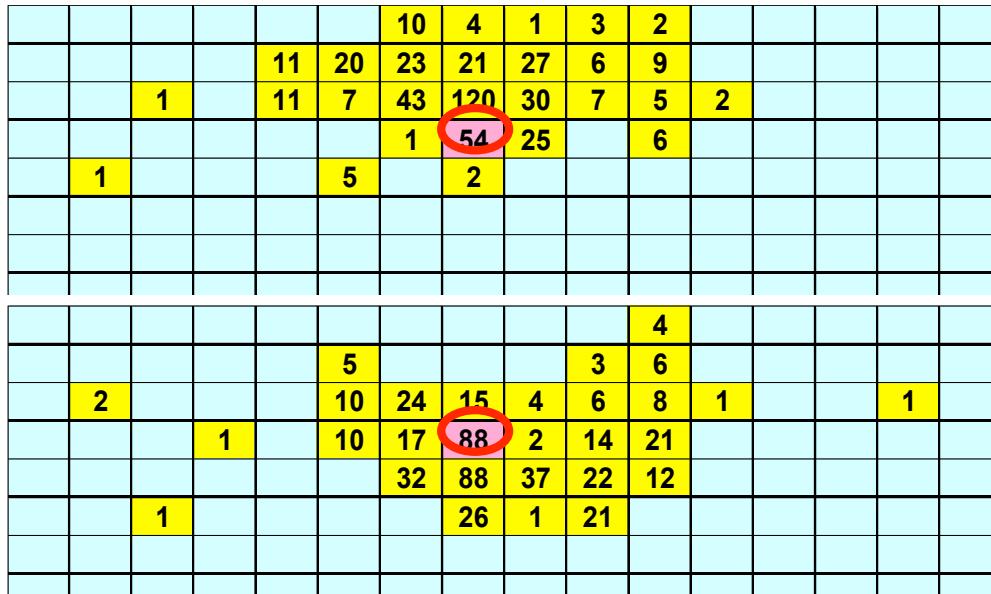


Fig. 4. Spread and incidence of bacterial spot lesions in tomato seedlings in two planter flats after infecting a centrally-located seedling (circled) with *Xanthomonas perforans*. Seedlings were sprayed daily with copper as Kocide.