BACTERIOPHAGES: A POTENTIAL BIOCONTROL AGENT AGAINST WALNUT BLIGHT (*XANTHOMONAS CAMPESTRIS* PV *JUGLANDIS*)

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ABSTRACT

During autumn 2000, isolates of bacteria were collected from blighted nuts, leaves, buds and petioles of walnut trees from all over New Zealand. From among these isolates *Xanthomonas campestris* pv *juglandis* lines were identified by a range of cultural, DNA fingerprinting and morphological tests. Concurrently, bacteriophages which attack *X. campestris* pv *juglandis* were isolated from the soils under the same trees. Phages were readily isolated from a depth of 2.5 cm. In the following spring phages were also isolated from the canopy of trees grown at Lincoln. Phage typing was carried out to produce a set of phages capable of destroying all bacterial isolates. A subset of the isolated phages was characterized. Several phages isolated from the soil were identified under the electron microscope as being from the λ phage group, with long unsheathed tails and hexagonal heads. Phages isolated from the canopy belonged to several other groups of phages. Short term storage had little effect on canopy phage survivability. Differences in hardiness were found between the phages. The search to locate effective, hardy phages for use as bio-bactericides continues.

Keywords: storage, ecology, bactericides, phage, phage-typing.

INTRODUCTION

Walnut blight, caused by the bacterium *Xanthomonas campestris* pv *juglandis*, is both a devastating disease of walnuts (Bull et al. 1985; Ramos 1998) and an excellent model system for a wide range of destructive plant bacterial diseases. At present copper sprays are used to control blight in walnut orchards (Ramos 1998). However, the bacteria are rapidly becoming resistant to copper sprays (Lee et al. 1994) and copper residues are poisoning our environment (Radix & Seigle-Murandi 1993). Alternative control chemicals are few, and even more toxic, while no effective plant resistance has been identified. A reliable and manageable biological control is needed. Bacteriophages provide highly specific control opportunities for bacterial diseases by specifically infecting and destroying the disease-causing bacteria. With no proven alternatives for walnut blight control, investigating the potential for bacteriophage based biocontrol is warranted.

MATERIAL AND METHODS

Bacterial isolations

Bacteria were isolated on nutrient agar from a range of infected walnut tissues collected throughout New Zealand. Individual colonies were purified by streaking under sterile conditions through several passages. Confirmation of isolates as *X. campestris* pv *juglandis*

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was by: 1) comparing with authenticated lines from Landcare Research, Auckland, 2) using the MicroLog computer identification program at the Geisenheim Research Institute, Germany, and 3) conducting a series of differentiating tests including, Gram stain reaction, microscopic examination, colony characteristics on GYCA agar, sensitivity to Triphenyltetrazolium chloride (Lovrekovich & Klement 1960), sensitivity to phages and characteristics on brilliant cresyl blue starch medium (Mulrean & Schroth 1981). Numbers of bacteria were counted in the buds using three replicates each of four buds per sample date. The buds were ground in either sterile water or nutrient broth and bacteria counted on selective media (Lindow et al. 2000).

Bacteriophage isolations and survival tests

Bacteriophages were isolated from the soil under walnut trees in the autumn of 2000 and from the canopy in spring of 2000. Methods of Crosse & Hingorani (1958) and Benson (1990) were used. An authenticated *X. campestris* pv *juglandis* isolate (134) was used for all phage isolations. Purified isolates were obtained by passage through at least three single plaque isolations. The phage solutions were filtered through 0.22 µm filters and stored at 4°C in sterile nutrient broth solution. Phage titres were measured by counting plaques formed in authenticated *X. campestris* pv *juglandis* lawns using standard dilution series methods in sterile water and the "double agar layer" method as described by Civerolo (1990). Phage typing was carried out as described in Benson (1990).

A set of bacteriophages purified and stored as indicated above had the number of plaque forming units counted (pfu/ml) at the start of storage and after 1 to 3 months storage. Replicated samples were counted.

Glasshouse trials

Branches of walnut cultivar Payne from a Lincoln University walnut trial were removed from the trees and placed in beakers of water in the greenhouse. The buds were sprayed either with water or a mixture of six soil phages in water (approximately 10^7 pfu/ml). Phages were re-isolated from the buds after 5 h using methods described above.

Electron microscopy

Samples of three of the phage solutions (P_2V_2 , P_8V_1 , P_{29}) were spun at 2500 g for 10 min. The supernatant was then spun at 49 000 g for 90 min and redissolved in 0.1 ml of sterile water. The samples, which contained in excess of 10^8 pfu/ml, were then examined using the transmission electron microscope at Landcare Research, Lincoln, using negative staining with 2% phosphotungstic acid (aqueous) at pH 6.5.

RESULTS AND DISCUSSION

Lytic bacteriophages of *X. campestris* pv *juglandis* were found to be widespread in the soils under walnut orchards throughout New Zealand. They were very easily isolated from the top 2.5 cm. Eight of 11 soil samples from the Lincoln University orchard yielded phages, as did 12 of 12 soil samples from other walnut growing sites in New Zealand. Three of the isolates examined using the electron microscope were not significantly different in size or form. All had long (181 ± 2 nm) unsheathed tails and hexagonal heads (76 nm long by 70 nm wide ± 2 nm). Attempts to make use of these soil bacteriophages as biocontrol agents were not successful. The mix of six soil phages applied to cut branches of Payne variety walnut trees in a glasshouse showed very poor survival. While application rate was approximately 10^6 pfu/bud, after only 5 h recoverable phage numbers had dropped to 111 ± 13 pfu/bud, which was not significantly different from untreated branches.

Figure 1 shows the environmental conditions during the spring and summer of 2000/2001, with the concomitant rise and fall in bacterial numbers in the buds of Payne walnut trees. Similar changes in bacterial numbers have already been demonstrated in US orchards (Haxo & Magnard 2000). There appears to be no great change in the environment associated with the fall in bacterial numbers per bud, and the maximum temperatures at the time of the decrease (26–28°C) have been found to be optimal for growing the bacteria in the laboratory.

FIGURE 1: Mean number of *X. campestris* pv *juglandis* on walnut buds (number of cells/bud) during spring (2000) and summer (2001), and the maximum air temperature (°C) and precipitation (mm) over that time.

TABLE 1: Variation in the *X. campestris* pv *juglandis* bacterial population and phage number during spring 2000 to summer 2001.

Date	Bacterial number (cells/bud) ¹	Phage number (pfu/bud) ²
12 Sep 2000	4 x 10 ²	1.1 x 10 ²
30 Oct 2000	3 x 10 ³	2.5 x 10 ³
8 Jan 2001	4 x 10 ²	3.4 x 10 ⁵

¹Mean of 16 samples. ²Mean of 2 samples.

There were substantial increases in phage numbers in the canopy as the bacterial populations fell after the start of December, suggesting there may be an involvement of phages in controlling blight bacteria in the canopy (Table 1). A number of phages were isolated from the canopy and characterised. Table 2 shows their effects on several *X. campestris* pv *juglandis* strains. Electron microscopy of these six phages indicated three different types, hexagonal with and without a short tail, and round. Thus phages in the canopy appear to be extremely variable in form and infectivity.

TABLE 2: Sensitivity response¹ of three different *X. campestris* pv *juglandis* strains to six bacteriophage types isolated from the walnut canopy.

Phage type	Bacterial strain		
	134 ² Lincoln	143 Lincoln	6494 Auckland
Bp60C ₁	+++	+	+
Bp60C ₂	+++	+	+++
Bp60C ₃	+++	+	+++
Bp ₁₀	+++	+	-
Bp ₂₀	+++	-	-
Bp ₂₂	+++	+	-

¹+++ Reactive (total lysis); - Not reactive; + Reactive (lysogenic).

²=Strain used for initial isolation.

Table 3 shows that the bacteriophages, particularly the canopy phages, survived well in the stored state and that there were differences in survivability among the phages. It therefore appears there may be a potential for using strains of the canopy phages as biocontrol agents. Trials looking at this will be conducted next spring.

TABLE 3: Percentage survival for six aerial phage types (*) after 1 month storage and for three soil phage types (#) after 3 months storage at 4°C.

	Phage type ¹	Initial Titre (pfu/ml)	Survival rate (%)
Bp60C ₁ *	C, NT	2.8 x 10 ⁸	71
Bp60C ₂ *	H, NT	2.5 x 10 ⁸	96
Bp60C ₃ *	H, ST	1.9 x 10 ⁸	85
Bp ₁₀ *	H, NT	2.7 x 10 ⁸	92
Bp ₂₀ *	C, NT	1 x 10 ⁵	68
Bp ₂₂ *	H, NT	1.7 x 10 ⁷	66
P ₄ V ₂ #	H, LT	10 ⁸	51
P ₇ #	H, LT	10 ⁶	62
P ₈ #	H, LT	10 ⁷	49

¹C = spherical, H = hexagonal, NT = no tail, ST = short tail, LT = long tail.

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