Survival and Spread of *Rhodococcus fascians* in greenhouse grown herbaceous perennials

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Introduction

Rhodococcus fascians is a Gram positive bacterium that infects up to 123 taxa of plants including many ornamentals important to the floral industry. The most commonly observed symptoms are shoot proliferation (Fig. 1a) and leafy galls (Fig. 1b). Little is known of the survival and transmission of the bacterium in greenhouse settings. The objectives of this study were to: 1) monitor survival of *R. fascians* strains on three different plant species known to be susceptible, 2) test the survival of *R. fascians* in irrigation water, 3) determine the role of irrigation water containing *R. fascians* as a source of inoculum for infection and 4) determine the spread of disease and movement of the bacteria from infected to healthy plants by overhead irrigation.

Figure 1a. *Nemesia* with shoot proliferation. 1b. *Gaura* with leafy gall

Methods and Results

Rifampicin resistant *R. fascians* strains used in these experiments were grown in a nutrient rich solid medium with the addition of 50 mg of rifampicin. Two to three day old cultures were transferred to liquid medium of the same composition and grown for two days on a rotary shaker at room temperature, then the suspension was adjusted to a final concentration of approximately 1 X 10^8 cfu/ml.

Survival of *R. fascians* on plant leaves. *Oenothera speciosa* 'Siskiyou', *Iberis gibraltarica,* and *Erysimum* 'Apricot Twist' were sprayed to run-off with suspensions of two strains of *R. fascians,* A25f and A44a, at a concentration of 10^8 cfu/ml. There were 8 plants sprayed with each bacterial strain and four control plants sprayed with sterile distilled water (SDW). One leaf was removed from each plant and suspended in 9.9 ml SDW on days 0 and 14. On day 28, leaves were suspended in 1 ml of SDW. Serial dilutions were made and spread to a rifampicin amended medium. The plates were incubated at 27º C and bacterial colonies were counted one week later to determine the concentration of bacteria on each leaf.

	Experiment 1		Experiment 2	
Plant host	Bacterial counts		Bacterial counts	
Bacterial strain	A25f	A44a	A25f	A44a
Day 0				
Erysimum	1.8×10^6	2.8×10^6	4.7×10^5	5.8×10^4
Iberis	1.3×10^6	3.5×10^6	4.9×10^5	5.0×10^3
Oenothera	5.6×10^6	1.0×10^6	1.7×10^6	6.4×10^4
Day 14				
Erysimum	3.7×10^3	$<1 \times 10^4$	$<1 \times 10^4$	$<1 \times 10^4$
Iberis	3.7×10^3	$<1 \times 10^4$	2.8×10^4	1.9×10^4
Oenothera	$<1 \times 10^4$	$<1 \times 10^4$	1.4×10^4	$<1 \times 10^4$
Day 28				
Erysimum	$<1 \times 10^3$	3×10^2	$<1 \times 10^3$	$<1 \times 10^3$
Iberis	2×10^3	2×10^2	3.6×10^4	3.1×10^3
Oenothera	1×10^2	2×10^3	4.8×10^3	4×10^4

Table 1. Average bacterial counts from eight leaves (cfu/ml) of plants inoculated by spraying a suspension of bacteria at 10^8 cfu/ml onto the foliage. No bacteria were detected from water sprayed controls.

	Experiment 1		Experiment 2	
Bacterial strain	A25f	A44a	A25f	A44a
Plant host	Number of leaves with detectable bacteria			
Day 0				
Erysimum	8	8	8	4
Iberis	8	7	8	2
Oenothera	7	8	8	7
Day 14				
Erysimum	2	0	0	0
Iberis	1	3	1	4
Oenothera	1	0	1	0
Day 28				
Erysimum	0	2	0	0
Iberis	1	1	1	2
Oenothera	1	1	4	2

Table 2. Number of leaves on which bacteria could be detected over time

Survival of *R. fascians* in water. Water was poured through three pots of *Oenothera* and 300 ml collected and pooled. The water was then spiked with *R. fascians* strain A25f resistant to rifampicin to a final concentration of 10^8 cfu/ml. The same procedure was followed for strain A44a to see how long the bacteria could survive in the absence of a host plant. In the first experiment the water was sampled daily for three weeks, then weekly; serial dilutions of sub-samples of the spiked water were spread to rifampicin amended media for bacterial counts until no more bacteria were detected. Bacteria were detected until day 60 with A25f and until day 92 with A44a (data not shown). The experiment was repeated three times with water samples being assayed every one to two weeks instead of daily. Experiments three and four were done with weekly, then semi-weekly samplings to show the progression of counts over time (Figures 5 and 6).

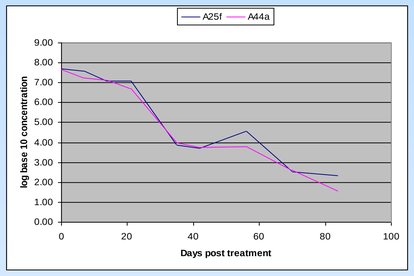

Fig. 5. Survival of bacteria in water poured through pots (Experiment 3). No colonies were detected when water was sampled on day 98.

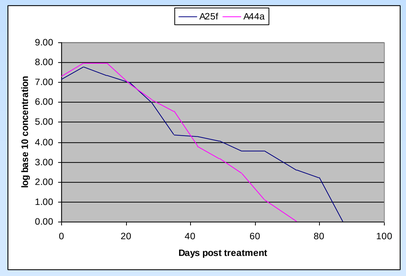

Fig. 6. Survival of bacteria in water poured through pots (Experiment 4). Counts were made from samples taken weekly.

Movement of bacteria off plants into water Ten *Oenothera* plants were sprayed to runoff with rifampicin resistant *R. fascians* strain A25f at a concentration of 10^8 cfu/ml. Pots were watered from the top only and placed over dishes so that irrigation water could be collected. An additional ten plants were sprayed with *R. fascians* strain A44a resistant to rifampicin. Four control plants were sprayed with water and all plants were maintained in a greenhouse. Samples of water from the dishes were collected weekly, immediately after plants were watered and as water accumulated in the dishes beneath the plants. Samples were serially diluted and spread to rifampicin amended media for plate counts. Water poured through pots after plants were sprayed to run-off with two strains of *R. fascians* contained detectable bacteria up through 50 days after treatment (Figures 8 and 9).

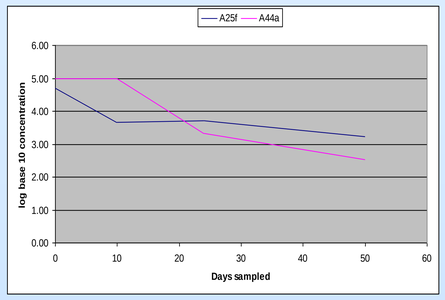

Fig. 8. Movement of *R. fascians* off leaves through soil over time. No samples were taken after day 50.

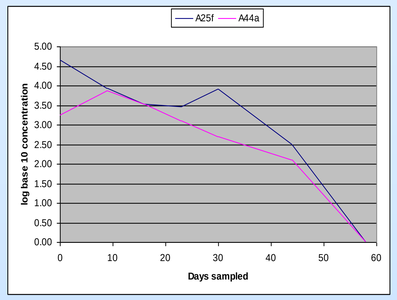

Fig. 9. Repeat of the experiment depicted in fig. 8; samples were taken until day 58 when no more bacteria were detected.

Infection of plants from irrigation water. *R. fascians* was added to saucers beneath 50 healthy *Oenothera speciosa* 'Siskiyou' plants. Four different strains of the bacterium, 02-815, A3b, A25f and A44a, at an inoculum level of 10^8 cfu/ml, were used. Ten additional plants were placed in saucers of uninoculated water as controls. This experiment was performed twice. Samples of leafy galls were removed from ten plants randomly selected on day 100 and put into 9 ml water blanks. The water was serially diluted and spread to plates amended with rifampicin to detect bacteria from the initial inoculum. Plates of semi-selective D2 medium were also streaked to detect *R. fascians*. Plant washes were used for PCR reactions to detect pathogenic *R. fascians* strains. In the first experiment, 23 of 50 plants developed leafy galls 100 days after bacteria was added to water in dishes below the plants. In the second experiment all 50 plants developed leafy galls (Table 3), and no leafy galls developed in 10 control plants.

Day of Observation	Plants with symptoms	Controls
0	0/50	0/10
7	0/50	0/10
21	0/10	0/10
35	9/50	0/10
49	9/50	0/10
65	24/50	0/10
79	43/50	0/10
89	43/50	0/10
100	50/50	0/10

Table 3. Symptom development on *Oenothera* after a mixture of four *R. fascians* strains was placed in saucers beneath the pots.

Spread of bacterial infection by water splash. Ten *Oenothera* plants were inoculated with 25 ml of *R. fascians* strain A25f resistant to rifampicin at a concentration of 10^8 cfu/ml. Another ten plants were inoculated with *R. fascians* strain A44a resistant to rifampicin. Four healthy, uninoculated plants were spaced from 2.5 to 10 cm around each inoculated plant. Plants were irrigated by overhead watering and observed for symptoms of leafy galls and/or shoot proliferation. The experiment was repeated. Results of the second experiment are shown in Table 4. Spread occurred as far as 10 cm from the original pot for both strains. Four control plants sprayed with SDW did not develop symptoms.

A25f		Distance of healthy plants from inoculated plant			
Day	Center pot (inoculated)	2.5 cm	5 cm	7.5 cm	10 cm
0	0	0	0	0	0
14	1	0	0	0	0
28	3	0	0	0	0
42	8	0	0	0	0
56	10	1	1	0	1
70	10	2	4	2	1
84	10	2	4	2	2
A44a					
0	0	0	0	0	0
14	4	0	0	0	0
28	6	1	0	0	1
42	8	1	1	3	1
56	9	2	4	3	3
70	9	3	5	4	3
84	9	3	5	4	3

Table 4. Spread of bacteria by water splash. Numbers in column two refer to number of plants showing symptoms of leafy galls.

Fig. 10 a. Arrangement of pots on greenhouse bench for water splash experiment. b. Spacing of four pots at 2.5, 5, 7.5 and 10 cm from center inoculated plant.

Discussion

In this study, using two isolates of *R. fascians*, bacteria were recovered from at least two different hosts as long as 28 days after inoculation. Although populations dropped dramatically over that time, it is important to note that the bacteria did not completely die off and could be recovered at 100-4,000 cfu/ml when leaves were incubated in water. It is likely that populations on infected greenhouse plants may sink to low levels, but should environmental condition become favorable for bacterial growth, populations could rebound. Infected plants appear to be a source of inoculum for healthy plants as far away as 10 cm. Symptom development is a long process, with symptoms at times not appearing until 70 days post inoculation. The experiments with longevity of *R. fascians* in water are illuminating as well. In the absence of a host, the bacterium can persist for over two months in regular tap water. Infection can occur when plants sit in infested water. Additionally, if inoculum is introduced to the aerial portion of the plant, *R. fascians* can be washed from the plant and can be recovered in leachate from the pots.

These experiments are the first documented investigations into movement of *R. fascians* in a greenhouse setting, and highlight the importance of strict sanitation in disease management. The long incubation period and ease of movement from plant to plant with standard greenhouse watering practices means infected plants should be discarded as soon as they are noticed. Adjacent plants should either be discarded or moved to a quarantine area where they can be observed for at least three months. *R. fascians* will persist in irrigation water, so plants must not sit in water on a bench or floor. These measures are especially important, as there are currently no known curative treatments for infected plants once the bacteria have been introduced into a production area.

Acknowledgements

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