PCR detection of pathogenic *Rhodococcus fascians* and *Agrobacterium tumefaciens* on herbaceous perennials

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Introduction

A wide variety of herbaceous perennials are susceptible to infections by *R. fascians and A. tumefaciens,* producing symptoms of leafy galls, shoot proliferation or tumors. These infections have had a major economic impact on the nursery industry in the past few years. Virulent and avirulent strains are found in both species, with genes for virulence found on an Ri or Ti plasmid respectively. Determining the causative agent for disease must therefore go beyond identification of the bacteria to include inoculations to indicator plants or detection of genes for virulence. It has often been difficult to isolate *R. fascians* from infected plants with leafy galls and the presence of Agrobacteria on these same plants has often led to confusing results. Misidentification of the infectious agent is possible if Agrobacteria are isolated but not tested for virulence. Only those bacteria that can reproduce the original symptoms can be confirmed as causing the disease. A rapid, sensitive and accurate method would be extremely useful for timely disease diagnosis. PCR has been used in a wide variety of plant diseases to detect pathogenic organisms.

The objective of this study was to develop PCR primers that would detect and discriminate between pathogenic *R. fascians* and A. *tumefaciens* strains.

Figure 1a. *Dicentra* with shoot proliferation (infected on left, control on right) **1b**. Leafy galls on *Oenethera* 'siskiyou'.

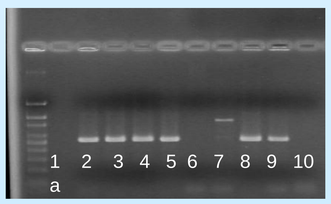
Figure 2a. *Heuchera* with leafy gall. **2b**. *Hibiscus* with crown gall.

Materials and Methods

Primer sets were designed from cytochrome p450 and *fas-1* region of the Ri plasmid in *R. fascians* and from the *Vir* region of the Ti plasmid in *A. tumefaciens*. These primers were used to amplify DNA from known virulent and avirulent strains in our culture collection as well as bacteria isolated from plants with tumors, shoot proliferation or leafy galls. Bacterial strains were grown on Mannitol Glutamate Yeast extract medium (MGY) for two days and a loopful of bacteria suspended in 0.75 ml sterile ultrapure water. DNA was extracted using InstaGene (BioRad, Hercules, CA).

For plants infected with *R. fascians*, 200 mg of tissue were suspended in 3ml of saline amended with 0.2 mg/ml cycloheximide for 24 to 48h at room temperature. For plants suspected of harboring *A. tumefaciens*, tumors were surface sterilized, then tissue within the tumor was cut into small pieces and placed into 3 mls of crown gall selective medium 1A. The samples were subjected to ultrasound for 1h, and left at room temperature for 24h. DNA was extracted from 1.5 ml of the plant suspensions using the InstaGene method. DNA templates were stored at -20C. The same plant wash methods were used to detect *R. fascians* from asymptomatic tissue on *Nemesia* and *A. tumefaciens* on *Argyranthemum.* PCRs were performed using 0.6 µl of DNA template in a 30 µl reaction. Gradient PCRs were performed to determine the highest possible annealing temperature to minimize non-specific priming. Universal 16s primers were used as positive controls to amplify a 1200 bp fragment of bacterial 16s rDNA genes.

Results



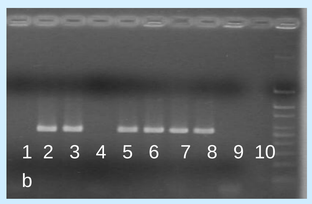


Figure 3a. *p450* products of *R. fascians* strains: lanes 2-5,8-9 are virulent (V+) and lanes 6 and 7 are avirulent (V-). **3b**. Lanes 2,3, 5-8 are V+ strains and lanes 4 and 9 are V-.

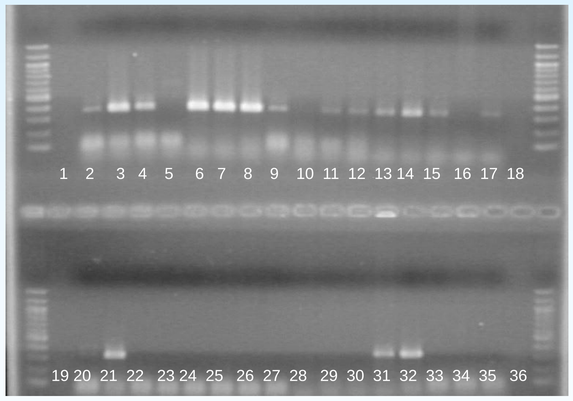


Figure 4. *VirB* products of *A. tumefaciens* strains: lanes 2-4, 6-17, 21, 31 and 32 are V+, lanes 5, 23-26 V-. Lanes 27-28, 31 are soil bacteria, lanes 29 and 30 are *Pseudomonas* sp. and 33 and 34 are *R. fascians*.

Table 1. Culture collection strains

Bacteria	Strains tested	*p450* product	*VirB* product
R. fascians			
virulent	13	13	0
avirulent	6	0	0
Agrobacteria			
virulent	29	0	26
avirulent	8	0	0
Corynebacterium	1	0	0
Erwinia	1	0	0
Microbacterium	3	0	0
Pseudomonas	5	0	0
Sphingobacterium	3	0	0
Soil bacteria	3	0	0

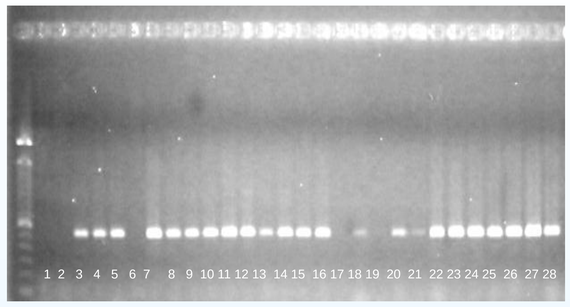


Figure 5. *p450* products of washes from plants inoculated with *R. fascians*. Lanes 2-18 *Oenothera*, lanes 19-20 *Iberis,* lane 21 *Nemesia,* lanes 22-28 *Erysimum*.

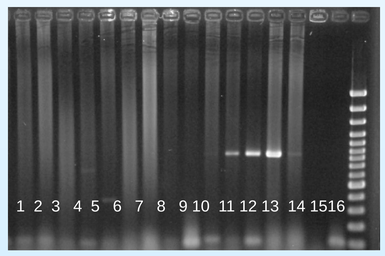


Figure 6. Plant washes from plants inoculated with *A. tumefaciens.* Lanes 11-12 *Bryophyllum*, lanes 13-14 *Iberis.* Lanes 2-10 are washes from *R.fascians* inoculated plants.

R. fascians was detected in 29 symptomatic plants inoculated with 10 different strains. The bacteria were re-isolated from *Oenothera, Iberis, Tiarella, Erysimum*, *Campanula*, and *Veronica*. The DNA extracted from these bacteria was amplified by the *p450* primers and produced bands of the same size on gels as the original bacterial DNA.

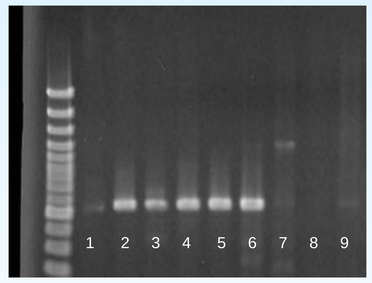


Figure 6. P450 products of plant washes from plants with leafy gall submitted to the OSU Plant Clinic. Lanes 1-3 washes from *Verbascum* 'Sierra Sunset,' lanes 4-6 plant washes from '6b. *Veronica* 'Royal Candles' and lanes 7-9 from *Verbena* 'Shawna Ann'.

Additional plant clinic submissions with leafy galls that produced a product with the p450 primers include *Aster, Campanula, Hosta, Lavatera, Leucanthemum, Nemesia*, and *Viola*. Plant washes from *Leucanthemum* and *Argyranthemum* plants with tumors produced a product when the *VirA* primers were used.

Figure 3a. *Nemesia* with shoot proliferation and **3b**. *Argyranthemum* with tumors. Plant washes were made from asymptomatic regions of both plants.

R. fascians was detected in plant washes from several sites on asymptomatic stems of *Nemesia* (Fig.3a) with the *p450* primers. Bacteria isolated from these areas were identified as *R. fascians* and also were positive with the *p450* primers. *A. tumefaciens* was similarly detected in plant washes from three asymptomatic stems and a flower bud of *Argyranthemum* (Fig. 3b) with the *VirA* primers. Bacteria isolated from these plant washes were identified as *A. tumefaciens* and were positive with the *VirA* primers.

Discussion

PCR was effective in amplifying DNA from pathogenic *R. fascians* and *A. tumefaciens* strains and detecting and discriminating between the two bacterial species in plant washes from a variety of plant hosts. Based on the results of this study, PCR should be an effective method for rapid diagnosis of crown gall or leafy gall in herbaceous perennials. PCR is also effective in detecting *R. fascians* and *A. tumefaciens* on asymptomatic plant material. Many nurseries rely on propagation by cuttings for rapid production of new plants. This could lead to widespread distribution of either bacterium before any symptoms are detected. PCR could be useful in screening stock plants to detect these two pathogens.

Acknowledgements

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