Development of Rapid Diagnostic Techniques for Detecting Bacteria that Cause Tumors, Shoot Proliferation and Leafy Galls in Nursery Plants

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Summary of Results

We investigated methods to improve recovery of *Rhodococcus fascians* from infected plants by varying the isolation medium and recovery methods. We found that shaking suspensions of *R. fascians* on a rotary platform for 22 hr enhances recovery when compared to other methods and thus could aid in recovery of *R. fascians* from plant washes. We also found that the use of pea seeds as bait does not enhance recovery of *R. fascians* and that alternative carbon sources had no beneficial effect on growth of *R. fascians*. We did find, however, that lowering the pH of our isolation broth to 6.0 improved growth of *R. fascians*.

We were interested in finding a host which would be reliable for testing pathogenicity of *R. fascians*. Garden peas were superior to sweet peas for pathogenicity testing. *Oenothera* reliably produced symptoms and is easily propagated in the greenhouse. We were able to successfully reproduce symptoms of shoot proliferation and leafy galls on plants used in the nursery trade, and found that there were differences in susceptibility between host species and some differences in the ability of various isolates to cause disease.

Finally, we developed PCR primer sets that allowed us to distinguish between pathogenic isolates of *Agrobacterium* and *R. fascians* in pure culture. This method subsequently was applied to washes of symptomatic plant tissue. These methods are now being used to diagnose *R. fascians* in samples submitted to the OSU Plant Clinic.

Introduction: In the past few years, wholesale nursery growers in Oregon have submitted numerous samples of herbaceous perennials with tumors, shoot proliferation or leafy galls to the OSU Plant Clinic. Initial isolations from symptomatic plants produced both *Agrobacterium tumefaciens,* the bacterium that causes crown gall, and occasionally, *R. fascians,* a Gram positive actinomycete. Although recovery of *R. fascians* was not frequent, the isolates we obtained were more consistently pathogenic than the *Agrobacterium* isolates. Bacteria recovered from plants were identified by the Biolog substrate utilization assay, which does not discriminate between pathogenic and non-pathogenic isolates, however.

Virulence of both *Agrobacterium* and *R. fascians* is conferred by plasmid-borne genes. Non-pathogenic isolates lack these plasmids, and may be intermixed among those that cause disease. All isolates recovered must undergo purification and inoculation to susceptible plants to determine if they are pathogenic.

Garden peas, the standard host used historically, were used to test pathogenicity of *R*. fascians. To determine if there was a more reliable host for pathogenicity testing, eight different plants were evaluated. Also, since indicator plants often show symptoms other than those found on the original host, we wanted to inoculate back to the same host from which we had obtained *R. fascians* to prove that bacterium was actually the cause of the symptoms.

Lastly, we wanted to find improved methods to isolate and detect, using molecular methods, pathogenic isolates of *Agrobacterium* and *R. fascians*. Since it is best to use more than one method to diagnose any given disease, we wanted to also try to improve upon traditional isolation methods.

Project objectives and results

Objective 1: Improve ability to isolate *Rhodococcus fascians* from symptomatic plants:

Experiment 1. Several treatments were tested in an attempt to increase recovery of *R. fascians* from symptomatic plants. Sonication (use of ultrasound) and agitation by mechanical shaker have been used successfully to improve the numbers of *Agrobacterium* cells recovered from plants with crown gall. We first examined the effects of these procedures on pure cultures of two isolates of *R. fascians* in D2 broth at a concentration of 10^8 cfu/ml. The treatments were: bath sonication of the culture for one hour, agitation on a shaker for 22 hours, a combination of sonication followed by agitation on the shaker, and incubation on the bench with no agitation or sonication. Serial dilutions were made after the four treatments and two plates spread with bacteria for each of three dilutions. All treatments resulted in a decline in viable cell density, however shaking the cultures resulted in the highest recovery (Table 1).

Table 1. Survival of *R. fascians* isolates A24a and A44a after sonication, shaking, both sonication and shaking, and after no treatment

	Cfu/ml	
	A24a	**A44a**
Sonicated	2×10^6	1×10^6
Shaken	1×10^7	6×10^7
Sonication + shaken	$< 1 \times 10^6$	$<1 \times 10^6$
Incubated only	4×10^6	1×10^6

Experiment 2. In early studies of *R. fascians*, pathogenicity was assessed by directly inoculating pea seedlings with infected plant material, without specifically attempting to isolate and purify the causal agent. An experiment was conducted to determine the effectiveness of enrichment with peas in the isolation process. Isolates A24a and A44a were inoculated to plants of *Erysimum* 'Bowle's Mauve' to establish a known infection. We tried to re-isolate the bacteria using pea seeds for enrichment by adding symptomatic plant material to D2 broth along with one of the six varieties of peas in Table 2, plus appropriate positive and negative controls. After 24 hours, serial dilutions were spread onto D2 plates and incubated at 27 C for seven days, then observed for colonies typical of *R. fascians*: orange, mucoid, flat colonies with entire margins. Colonies typical of *R. fascians* were recovered from *Erysimum* incubated with 'Laxton Progress' peas, 'Elegance' sweet pea, 'Mrs. Collier' sweet pea, and D2 alone (data not shown). 'Early Multiflora' sweet pea and 'Tall' pea added to the D2 did not result in growth of any typical colonies. We concluded that there was no apparent benefit from the addition of peas to D2 isolation medium.

Experiment 3. Another potential way to improve recovery of *R. fascians* is to increase the selectivity of the D2 medium, which is only weakly semi-selective. D-(-) mandelic acid, Tween 40, and Tween 80 are utilized by *R. fascians* but did not improve growth compared to the standard D2 medium.

Experiment 4. Indications in the scientific literature are that pH may have an effect on growth of *R. fascians*. We compared the growth of *R. fascians* A25f and A44a in D2 medium adjusted to pH 5.0, 6.0 and 7.8, the latter being the normal pH of the D2. Standardized suspensions of bacteria were incubated in shaken flasks and the turbidity was measured every hour for eight hours, then every two hours until 10 PM. Final readings were taken the following morning. Both A25f and A44a grew faster and to a higher concentration in the medium at pH 6.0 than at pH 5.0 or 7.8. The experiment was

repeated with similar results. Therefore, the pH of D2 now used for isolation is adjusted to pH 6 instead of 7.8.

Objective 2: Determine which plant hosts are most suitable for pathogenicity testing.

Experiment 1. Sweet pea (*Lathyrus odoratus*) was the first host on which *R. fascians* was detected and described, and has been used successfully as an indicator host in screening *R. fascians* isolates for pathogenicity. We wanted to look for potential differences in symptom expression in various cultivars of sweet and garden peas. . Sweet peas 'Elegance', 'Mrs. Collier', and 'Multiflora' and the garden pea (*Pisum sativum*) 'Laxton Progress', 'Sugarpod,' and 'Tall' were evaluated. Seeds were washed for one hour in running tap water, surface sterilized by immersion in 10% bleach for 15 minutes, rinsed for another 30 minutes in running tap water, then placed in sterile Petri dishes with two layers of filter paper moistened with sterile distilled water. For each treatment, ten germinated seeds were soaked for two hours in a bacterial suspension of 10^8 cfu/ml of one of four different *R. fascians* isolates. The seeds were planted in 6-inch pots, placed in the greenhouse, and observed over a two month period. As seedlings emerged, many exhibited distorted growth and multiple shoots, typical of symptoms first reported on sweet peas infected with *R. fascians* (Figure 1). A comparison of incidence of symptoms on the six pea varieties is shown in Table 2. Even though all varieties responded to the inoculations, sweet peas have a much thicker seed coat, take longer to germinate, and are more expensive than garden peas. The garden pea varieties 'Laxton Progress' and 'Sugarpod' were used in all subsequent inoculations when testing for pathogenicity of *R. fascians* isolates.

In a separate, subsequent experiment, we found that symptoms appear on *Oenothera* as early as two weeks after inoculation (data not shown), and we will use this host preferentially for all future inoculations.

Figure 1. Pea seedlings inoculated with *A. fascians* strain A78. Note the multiple small shoots.

Table 2. Comparison of sweet peas and garden peas for expression of pathogenicity of *R. fascians* isolates.

Number of symptomatic/Number of surviving seedlings.

***R. fascians* isolates**	02-815	A25f	A44a	A76
Pea variety				
'Elegance'	2/6[a]	6/6	5/5	6/8
'Mrs. Collier'	4/5	5/6	2/4	5/5
'Multiflora'	4/5	4/4	5/5	3/3
'Laxton Progress'	4/5	3/3	2/5	7/7
'Sugarpod'	5/5	3/3	3/3	0/1
'Tall'	0/2	2/2	0/0	5/5

Objective 3: Establish causality in plants showing shoot proliferations and leafy galls by fulfilling Koch's postulates.

Experiment 1. A wide range of plants were inoculated with two tumorigenic *A. tumefaciens* strains and four *R. fascians* strains. Known pathogenic strains of both bacterial species served as positive controls. Negative controls consisted of water treated plants. Plants were evaluated weekly for four months. Results are shown in Table 3. One isolate, A44a, produced shoot proliferations and/or leafy galls on nine of the 16 hosts. The three other *R. fascians* strains produced symptoms of the same type, but on fewer host plants, indicating that there are differences in the ability of *R. fascians* isolates to cause disease. There was also a differential host response between members of the same genus. For example, the *Iberis gibraltarica* produced both shoot proliferation and leafy galls, whereas *Iberis* 'Alexander's White' appeared resistant. Inoculation with *A. tumefaciens* resulted in tumors on four of eight hosts inoculated. At no time did we see leafy galls or shoot proliferation in plants inoculated with *A. tumefaciens*.

Table 3a. Host range inoculation results: *Rhodococcus fascians*.

R. fascians isolate used (Original Host)	A3b (*Heliopsis*)	A25f (*Nemesia*)	A44a (*Veronica)*	02-815 (*Campanula*)
Plant inoculated	**Reaction of inoculated plant**			
Bryophyllum diagremontiana	None	None	None	None
Campanula 'Birch hybrid'	None	None	None	None
Campanula medium 'Pink'	SP	SP	SP	SP
Echinacea 'Magnus'	None	None	None	None
Erysimum 'Apricot Twist'	SP	SP	SP	SP
Erysimum 'Bowle's Mauve'	SP	ND	SP	SP
Erysimum 'Pastel Patchwork'	SP	SP	SP, LG	SP
Erysimum 'Variagata'	SP	SP	SP	SP
Hosta 'Whirling Dervish'	None	None	None	None
Iberis 'Alexander's White'	None	None	None	None
Iberis gibraltarica	SP, LG	SP, LG	SP, LG	SP, LG
Nemesia	None	SP, LG	SP, LG	None, LG
Oenothera 'Siskiyou'	LG	LG	LG	LG
Penstemon	None	None	None	None
Symphyotricum	None	None	None	None
Tiarella	None	None	SP	None
Veronica 'Blue Fox'	None	None	SP	None

LG = Leafy gall T = Tumor SP = Shoot proliferation None = No symptoms

Table 3b Host range inoculation results: *Agrobacterium tumefaciens*		
A. tumefaciens isolate used (Original Host)	A47a (*Achillea*)	A91-1 (*Dendrathema*)
Plant inoculated	**Reaction of inoculated plant**	
Bryophyllum diagremontiana	T	T
Echinacea 'Magnus'	T	T
Erysimum 'Bowle's Mauve'	T	T
Hosta 'Whirling Dervish'	None	None
Iberis 'Alexander's White'	ND	NS
Iberis gibraltarica	T	None
Oenothera 'Siskiyou'	None	None
Penstemon	T	T
T = Tumor SP = Shoot proliferation None = No symptoms ND = Not done		

Objective 4: Develop PCR methods for detecting and discriminating between pathogenic *A. tumefaciens* and *Rhodococcus fascians* strains on symptomatic plants.

The polymerase chain reaction (PCR) is a diagnostic tool that can identify a specific stretch of DNA. It is widely used in the diagnosis of plant and animal diseases. The first step is to extract DNA from the samples in question. This DNA is added to a mixture that contains primers (short stretches of DNA that have sequences that exactly matches the DNA sequence to be detected) and other reagents that allow amplification of the target sequence, if it is present in the DNA preparation.

Isolation of template DNA from cultures. A representative set of cultures was grown for three days on three different media. Template DNA was extracted from these cultures by one of two methods: a boiling method where bacterial cells from pure cultures were boiled to release the DNA; and a method using a commercial kit (InstaGene, from Bio-Rad, Hercules, CA). The InstaGene preparations proved to be more reliable as PCR templates. For example, the InstaGene method produced DNA suitable for PCR reactions in 29 of 29 preparations whereas the boiling method produced suitable DNA in 21 of 29 preparations from the same set of cultures. The InstaGene preparations were also superior in regards to storage at -20 C. Cultures grown on any three of the media were suitable for template preparations.

Development of PCR primers and reaction conditions. We devised a primer set that specifically detects the p450 gene that is required for virulence in *R. fascians* and a primer set that specifically detects the *vir*A gene that is required for pathogenicity in *Agrobacterium*. It is important to include a positive control to evaluate PCR results, particularly in the case of a preparation that yields a negative result with the diagnostic primers. If a given preparation tests negative using the diagnostic primers, it must be shown to contain bacterial DNA that can serve as an effective template in a PCR assay. For this we used a primer set that will universally detect the 16S rDNA gene that all bacteria have. The 16S primed PCR serves as a quality control check for the DNA preparation, and as a positive control for the PCR assay. The PCR conditions were optimized by varying annealing temperature, extension time, and amount of template. The efficacy of this assay was tested with all three primer sets and template preparations from 23 different isolates of *R. fascians*, 7 different isolates of pathogenic *Agrobacterium*, and two unrelated bacteria also isolated from leafy galls (*Ochrobactrum anthropi* and *Stenotrophomonas maltophilia*). In all cases, the p450 primers specifically detected the *R. fascians* isolates (positive for p450, negative for *vir*A, positive for 16S), and the *vir*A primers specifically detected the pathogenic *Agrobacterium* (negative for p450, positive for virA, positive for 16S). The unrelated bacteria only tested positive for 16S. Representative PCR products are shown in Figure 2.

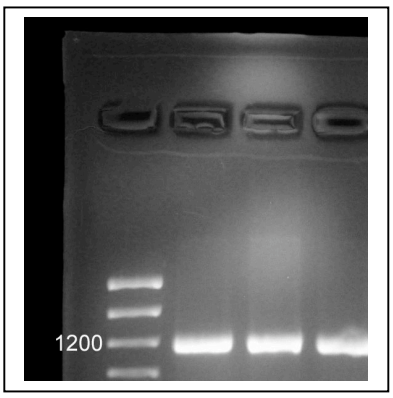

Figure 2. 16S, p450and *vir*A PCRs were done separately; the 16S reactions were combined with their respective p450 or *vir*A reactions and visualized on this ethidium bromide-stained gel. Lane 1 is a 100 base-pair (bp) ladder of DNA size standards. Lanes 2 and 3 are PCR products from cultures of *R. fascians*; the large 16S product (top band) and the 538 bp p450 product (lower band) are visible. Lane 4 is from a culture of a pathogenic *Agrobacterium.* The large 16S product and the 850 bp *vir*A product are visible. Lanes 5 and 6 are from two different plant samples submitted to the OSU Plant Clinic. Lane 5 clearly shows the *vir*A product indicative of pathogenic *Agrobacterium,* whereas lane 6 clearly shows the p450 marker indicative of pathogenic *R. fascians*.

Detection of *R. fascians* and *Agrobacterium* in symptomatic plant material. A variety of plants (cultivars of *Oenothera, Campanula, Erysimum, Tiarella, Veronica*, and *Iberis*) were inoculated (in duplicate or triplicate) with 10 different pathogenic isolates of *R. fascians* to form 19 different bacteria-plant combinations. Similarly, plants of *Iberis* and *Bryophyllum* were inoculated with various pathogenic isolates of *Agrobacterium*. Samples of symptomatic tissue were suspended in sterile saline amended with 100 mg/l cyclohexamide and incubated at room temperature for 24 to 72 hours. Samples of this saline wash were treated with the InstaGene matrix then assayed with the p450 (virulence

marker for *R. fascians*), *vir*A (virulence marker for pathogenic *Agrobacterium* sp.) or universal bacterial 16S rDNA primers (quality control check) in PCR reactions.

Overall, 61 of 62 DNA preparations from symptomatic plants tested positive for the 16S marker. This indicates that we were successful in obtaining suitable DNA 98.4% of the time. In these reactions, we also successfully detected pathogenic *R. fascians* in 53 of 55 (96.4%) samples of symptomatic plants. Because we had performed duplicate sampling, *R. fascians* was effectively detected in every symptomatic plant tested (29 different plants). All samples also correctly tested negative for the *vir*A marker indicative of pathogenic *Agrobacterium*.

Two plant species were inoculated with four isolates of pathogenic *Agrobacterium*. The six samples taken from these plants were all positive for the virA marker indicative of pathogenic *Agrobacterium* and negative for the p450 marker indicative of *R. fascians*. In conclusion, the combination of *vir*A and p450 primer sets used in our PCR reactions consistently detected pathogenic *R. fascians* in symptomatic plants and excluded the possibility of infection with pathogenic strains of *Agrobacterium*. These procedures have been applied effectively to detect *R. fascians* in 8 different OSU Plant Clinic submissions, and to detect pathogenic *Agrobacterium* in two different OSU Plant Clinic submissions

In summary, PCR was used to detect *R. fascians* in 29 of 29 symptomatic plants which had been inoculated with various isolates of *R. fascians.* Similarly, pathogenic strains of *Agrobacterium* were identified from plant samples. Several procedural details have contributed to the effectiveness of this assay. First, duplicate samples were taken from each plant. Secondly, the DNA template preparations were tested using two different amounts in the PCR assays. This is because the effective template concentration is unknown, and different volumes may be needed to minimize the potential for inhibition by plant compounds. Finally, the use of the 16S primers as a quality control check allows the identification of the rare DNA preparation that is ineffective as a PCR template.

Conclusions: We report that all four objectives have been met. (1) We improved the methods for isolation of *R. fascians* from symptomatic plant material. (2) We identified new hosts for *R. fascians*, found that various host plants had different symptomology, and identified *Oenothera* as a better host for testing than peas. (3) We fulfilled Koch's postulates. (4) We developed a rapid PCR assay to identify *R. fascians* in symptomatic plants.