

PCR Assay of the Rhizosphere Soil of Weeds Associated with an Outbreak of Bacterial Wilt of Ginger in East Hawaii.

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ABSTRACT

Weeds were collected from a ginger field experiencing a bacterial wilt outbreak. These weeds were assayed for the presence of the pathogen (*Ralstonia solanacearum*) using a PCR assay. The pathogen was detected from the rhizosphere soil of many but not all samples. We hypothesize that weeds that consistently harbor high populations of this bacterium may serve as an alternate host, and indicator plant for, this pathogen.

INTRODUCTION

Ralstonia solanacearum (Smith) (Yabuuchi, Kosako *et al.* 1995) is a bacterial plant pathogen of global importance, especially in the tropics and subtropics (Buddenhagen and Kelman 1964). This pathogen can be classified into 5 races (defined by host range) that altogether can infect more than 450 plant species (Hayward, 1991). *R. solanacearum* strains that cause disease in the plant family Zingiberaceae, including *Zingiber officinale*, edible ginger, is designated as race 4 (Schaad, Jones *et al.*, 2001).

Bacterial wilt of ginger is an important production constraint for edible ginger production in Hawaii. This disease was first reported in Hawaii in 1964 (Ishii and Aragaki 1963), but more recent outbreaks have been much more severe. As an example, an epidemic of bacterial wilt of ginger reduced the yield of Hawaiian ginger in 1992-1993 by more than 60% (Rohrbach *et al.* 1993). This represents a yield loss of approximately 9 million pounds, and a local revenue loss of \$5 million. Similar losses (approximately 50%) were experienced in 1994-1995, and significant losses have been reported every year since. Major factors in this epidemic, as in all bacterial wilt epidemics, include the unintentional use of infected planting material and planting in infested fields (Hayward 1991).

This bacteria is persistently soilborne and is easily disseminated via water, soil and cultivating equipment. Thus, once a field is infested with the pathogen, it is unsuitable for ginger cultivation and serves as an inoculum source for fields subject to runoff as well as for recipients of planting material and cultivation equipment. Understandably, ginger growers avoid fields known to have had a bacterial wilt outbreak and move on to virgin (with regard to ginger) fields. This practice leads to a persistent yearly expansion of infested acreage. Attempts to reclaim an infested field for ginger cultivation can result in early crop failure, even if no ginger or tomato had been grown for 10 years or more (personal communications from growers).

A reliable method to detect this pathogen would be a useful tool to evaluate planting material and fields under consideration for ginger cultivation. Others have recently reported on detection methods for *R. solanacearum* from various substrates (Weller, Elphinstone *et al.* 2000) (Schonfeld, Heuer *et al.* 2003) (Seal, Jackson *et al.* 1993) (Horita, Yano *et al.* 2004) (Seal, Taghavi *et al.* 1999). These procedures all use variations of the polymerase chain reaction (PCR). Previously, we reported on a polymerase chain reaction (PCR) assay for detection of *R. solanacearum* (Shintaku, Kaneshiro *et al.* 1996). We showed that

this assay was very sensitive, and suitable for use in assaying ginger seedpieces. We are currently developing a PCR-based assay to detect *R. solanacearum* in weeds and soil, in order to evaluate fields before cultivation as well as to identify weeds that serve as alternate hosts for this pathogen. Towards that end, we evaluated a soil extraction procedure for use in PCR-based tests. Our aim is to use PCR to track changes in the population of this pathogen when associated with various weed hosts to first of all identify alternate hosts, e.g. hosts that can sustain populations of the bacteria either in its rhizosphere or plant tissue, without becoming diseased. After identifying such hosts, evaluation of a field for potential ginger cultivation would include an assay of weeds present in the field. If known alternate hosts of *R. solanacearum* are present, they can be assayed by PCR, and should populations of *R. solanacearum* occur the field can be avoided.

We report here on an assay conducted on weeds within a bacterial wilt-affected ginger field. Our protocol was to sample weeds and ginger from affected areas, and to collect soil associated with the rhizosphere of these weeds. We extracted total DNA from these soil samples, and then used *R. solanacearum*-specific primer sets to identify samples containing *R. solanacearum*. Previous studies have shown that extracting DNA for PCR analysis can be difficult and time-consuming (Young, Burghoff *et al.* 1993) (Pillai, Josephson *et al.* 1991), and for this study we used a commercial soil DNA extraction kit (UltraClean Soil DNA kit™ MoBio Inc., Carlsbad, CA). We obtained good results using this kit, and the DNA extracted from many of our soil samples tested positive by PCR for the presence of *R. solanacearum*. We identify several weeds that have populations of *R. solanacearum* within their rhizosphere, and these are potential alternate hosts.

MATERIALS AND METHODS

Sample collection

Samples were collected in March and August 2005, from an East Hawaii ginger field belonging to a farmer reporting bacterial wilt-related symptoms. Weed samples were collected in areas containing symptomatic plants. Collection was performed by pulling plants out of the soil and transferring them to sealable plastic bags. Ginger samples were also collected.

Bacterial isolation and maintenance

Ginger rhizomes from symptomatic plants were dissected, and where ooze was evident, it was streaked onto PT-M2 (Nishijima, Alvarez *et al.* 2004) plates. The dissected pieces were also submerged in distilled water, and the water then tested for *R. solanacearum* with a *R. solanacearum* ImmunoStrip™ test (Agdia, Inc., Elkhart, IN). After incubation at 28°C, colonies appearing on PT-M2 plates were subcultured in order to obtain pure cultures. These pure cultures were tested using a PCR test (Shintaku, Kaneshiro *et al.* 1996) as well as the ImmunoStrip™ test to confirm the presence of *R. solanacearum*. These cultures were harvested from plates and stored in 50 ml tubes in sterile distilled water at room temperature.

Soil DNA extraction

In the laboratory, bags containing the weeds were shaken to release soil from the roots and then these soil samples were processed for DNA extraction. DNA extraction was performed using the UltraClean Soil DNA kit™ (MoBio Inc., Carlsbad, CA) on 0.5 gram samples of soil. After processing, all DNA samples were stored at -20°C.

PCR and electrophoresis

PCR was performed on soil DNA samples collected in March 2005 was conducted in 25 μ l reaction volumes using the OLI-1 (Seal, Jackson *et al.* 1993) and Y2 (Young, Downer *et al.* 1991) primers, with buffer, nucleotides and GoTaq® DNA polymerase purchased from Promega, Inc. located in Madison, WI. Thermal cycling was carried out in an Eppendorf MasterCycler personal thermal cycler (Eppendorf, Inc., Hamburg, Germany), with the following program: 95°C 15 sec., 50°C 60 sec., and 72°C 30 sec for 35 cycles.

PCR on soil DNA samples collected in August 2005 was conducted in 10ul reaction volumes using the primers AKI-R and AKI-F (Horita, Yano *et al.* 2004), and using Immomix Red (Bioline, Inc., Boston, MA), as the PCR reagent master mix. Thermal cycling was conducted using the following program: initial 94°C 7 min., followed by 94°C 15 sec., 61°C 30 sec., and 72°C 30 sec, for 35 cycles.

Agarose gel electrophoresis was carried out in a 1% or 1.5% agarose (MP Biomedicals, LLC, Aurora, OH) Tris-acetate-EDTA gel, in a Mini-Wide submarine cell (BioRad Inc., Hercules, CA). All gels were stained with either ethidium bromide or SYBR-safe (Invitrogen, Inc., Carlsbad, CA).

RESULTS AND DISCUSSION

All weed samples were taken from field sections known to be affected with *R. solanacearum*. Positive PCR results from rhizosphere samples from these weeds were obtained in numerous but not all samples examined (Figure 1, Table 1). This indicates that the soil DNA extraction method we used was adequate for obtaining DNA for PCR analysis. In addition, we obtained very strong positive signals (read as abundant DNA in the amplified fragments) in some samples. While the PCR is not considered a quantitative test, a very strong signal in a test such as this one indicates an abundance of target DNA and thus an abundance of targeted bacteria (in this case *R. solanacearum*).

This study includes 47 specimens from 13 weed species. Some weeds in the sampled area were more abundant than others and this is reflected in the numbers of the collected specimens. Interestingly, the rhizosphere of the *Phyllanthus sp.* samples, and to a lesser degree the *Erechtites sp.* samples we collected consistently yielded strong PCR signals, indicating high populations of *R. solanacearum*. These, along with some of the other weeds tested, are potential maintenance hosts of this pathogen. These weeds, especially the ones multiply sampled, are very common in the East Hawaii ginger-growing area and are thus excellent candidates for assay plants. We are initiating greenhouse studies using these weeds to establish the persistence of the association of *R. solanacearum* with, in particular, *Phyllanthus sp.* and *Erechtites hieracifolia*.

Our goal is to use the plants identified in these studies as indicator plants so that fields under consideration for ginger cultivation can be assayed for the presence of *R. solanacearum*. Random sampling of soil may miss the pathogen. As an example, the soil at our experimental farm (UHH CAFNRM Agricultural Farm) is infested with *R. solanacearum* as evidenced by disease occurrence whenever tomato is planted there, yet ELISA and PCR tests of random soil samples routinely test negative for the pathogen. If the pathogen is maintained by particular weeds, this would target our sampling of potential soils to likely pockets of this pathogen.

Table 1 *R. solanacearum* presence (determined by PCR) in the rhizosphere of weed hosts harvested from an affected ginger field. An amplified DNA product indicates the presence of *R. solanacearum*

Weed sampled	Common name	PCR result
<i>Cardamine sp.</i>	bittercress	+
<i>Centella asiatica</i>	spadeleaf	-/++
<i>Crepis sp.</i>	hawksbeard	-
<i>Cymbopogon refractus</i>	barbwire grass	+
<i>Cyperus hypochlorus</i>	Oahu flatsedge	+
<i>Digitaria sanguinalis</i>	hairy crabgrass	-
<i>Emilia sonchifolia</i>	lilac tasselflower	-/-/+
<i>Erechtites hieracifolia</i>	burnweed	-/-+/+/+/+/+/+/+/+
<i>Ipomea sp.</i>	morning glory	-
<i>Kyllinga sp</i>	spikesedge	-/-/+/+/+
<i>Panicum maximum</i>	Guinea grass	-
<i>Phyllanthus sp.</i>	leafflower	-/-/+/+/+/+/+/+/+
<i>Pluchea indica</i>	Indian camphorweed	-/-/-/+
<i>Solanum nigrum</i>	black nightshade	-/-/-/-/+/+/+/+/+

Where multiple samples of a weed species were assayed, the individual results are separated by /'s.

- No *R. solanacearum* detected
- + *R. solanacearum* detected
- ++ *R. solanacearum* detected (very strong signal, see Figure 1)

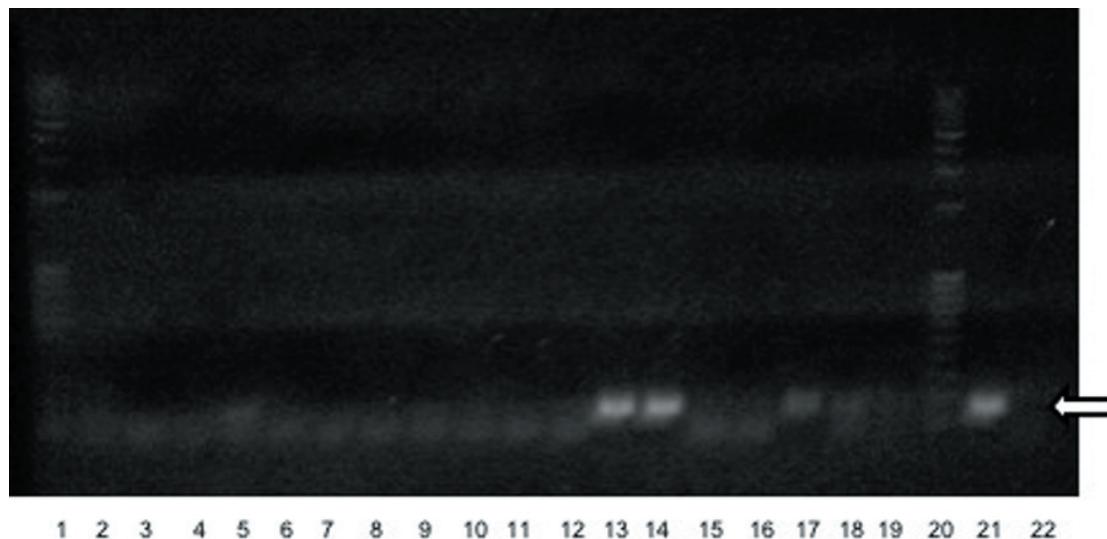


Figure 1.

Example of electrophoresis analysis of PCR products The primers AKI-F and AKI-R were used in this assay. The arrow on the right indicates the position of the amplified band from *R. solanacearum*. The fragment size is 165 bp., and bands indicating a positive result occur in lanes 5, 13, 14, 17, 18 and 19. Lane 21 contains the positive control. Lanes: 1 and 20-molecular weight markers; 2 through 5-P. niruri; 6 and 7-unidentified weeds; 8-Indian fleabane; 9-unidentified; 10-C. asiatica; 11 through 14-S. nigrum; 15-unidentified;16 through18-E. hieracifolia;19-unidentified; 21-positive control (*R. solanacearum* from ginger); 22-negative control

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