Ralstonia solanacearum Race 4: Risk Assessment for Edible Ginger and Floricultural Ginger Industries in Hawaii

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ADDITIONAL INDEX WORDS. bacterial wilt, *Zingiber officinale*, *Hedychium gardenarium*, biological control agent

SUMMARY. Fourteen species of ginger belonging to Zingiberaceae and Costaceae were evaluated for susceptibility to the bacterial wilt pathogen *Ralstonia solanacearum* (Rs) race 4 (ginger strains) by several methods of inoculation, including tests to simulate natural infection. Twelve of 14 species tested were highly susceptible to all strains of Rs race 4 upon stem inoculation, and susceptible plants wilted within 21 days. In contrast to previous reports that Rs strains from an invasive alien species, kahili ginger (*Hedychium gardenarium*), are nonpathogenic on ornamental gingers, the kahili ginger strain wilted both ornamental and edible ginger (*Zingiber officinale*) species within 21 days. Pour inoculation to the base of 11 plant species to simulate natural infection confirmed the ability of Rs to invade all the tested species without root wounds. Shampoo ginger (*Zingiber zerumbet*) was the most susceptible (wilted in 26 days) whereas pink ginger (*Alpinia purpurata*) and red ginger (*A. purpurata*) were the least susceptible and wilted in 71 and 76 days respectively. Pathogen survival in potting medium was evaluated by enumerating viable cells in effluent water from drenched pots with and without infected edible ginger after stem or rhizome inoculation. *Ralstonia solanacearum* survived in plant-free potting medium for 120 days and for 150 to 180 days in potting medium with infected edible ginger. The ability of Rs race 4 to infect many ginger species without wounding and to survive for long periods indicates that high risks will be incurred if the kahili ginger strain is inadvertently introduced from the forest reserves into ginger production areas.

Bacterial wilt, caused by *Ralstonia solanacearum*, causes severe wilt in many crops and is widely distributed in tropical, subtropical, and temperate regions of the world (Hayward, 1991, 1994; Kelman, 1953). *Ralstonia solanacearum* is classified into five races based on the hosts affected, and five biovars based on the ability to use or oxidize several hexose alcohols and disaccharides (Buddenhagen et al., 1962; Hayward, 1964). Race

1 strains (biovars 1, 3, and 4) are pathogenic to a broad range of hosts, including tomato (*Solanum lycopersicum*), tobacco (*Nicotiana tabacum*), and peanut (*Arachis hypogaea*); race 2 strains (biovars 1 and 3) infect banana (*Musa acuminata*), plantain (*Musa paradisiaca*), heliconia (*Heliconia* spp.), and other plants in the Musaceae family; race 3 strains (biovar 2) occur in cool upland areas in the tropics and cause severe wilt in potato (*Solanum tuberosum*), tomato, and geranium (*Geranium* spp.); race 4 strains (biovars 3 and 4) infect ginger; and race 5 strains infect mulberry (*Morus alba*). The strains in the race 3 group are a select

agent under the U.S. Agricultural Bioterrorism Protection Act of 2002 (U.S. Department of Agriculture, 2005). Pathogen diversity and the relationship among races, biovars, and phylotypes have been addressed recently (Alvarez, 2005; Fegan and Prior, 2005).

Edible ginger is a major vegetable and spice crop in Hawaii and in numerous locations in Australia, India, Indonesia, Jamaica, Japan, Malaysia, Nigeria, Sierra Leone, and the Philippines. Hawaii's farmers harvested 4.3 million lb of edible ginger during the 2005–06 season and the total farm value was estimated at \$3.0 million (Hudson, 2006b). Plants belonging to the Zingiberaceae and Costaceae families are prevalent in the forests of Hawaii and are grown for cut flowers as well as for the "lei" industry, which is important because of its aesthetic and symbolic value to traditional customs and the local tourism industry. Hawaii's floriculture industry includes numerous economically important gingers from Zingiberaceae and Costaceae. The value of ginger cut flowers in Hawaii was \$1.61 million in 2005–2006 (Hudson, 2006a), the predominant flowers being red ginger and pink ginger.

Bacterial wilt disease of edible ginger causes severe economic damage in many countries, including China, India, Indonesia, Japan, Malaysia, Mauritius, the Philippines, and the United States (Hawaii) (Alvarez et al., 2005; Kumar and Sarma, 2004). In Hawaii the disease was first reported on the island of Oahu in the early 1960s (Ishii and Aragaki, 1963; Quinon et al., 1964). Crop losses of edible ginger resulting from bacterial wilt exceeded 50% in 1998 and 1999 (Yu et al., 2003), and in 2005, the area harvested was reduced by 13% (Hudson, 2006b). In India, bacterial wilt is widespread on edible ginger and 100% yield losses have been reported (Dohroo, 1991; Mathew et al., 1979; Sarma et al., 1978;

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This research was funded by the USDA Special Grants program for Tropical and Sub-tropical Agricultural Research (Award no. 2004-34135-15191) and USDA-ARS/Minor Crops Research (Award no. 59-5320-1-525).

We thank E.E. Trujillo, D.E. Gardner, M.T. Momol, and D. Cook for providing us with bacterial strains; and C.W. Morden and R.F. Baker of Lyon Arboretum, University of Hawaii at Manoa, for providing planting stocks.

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Units

To convert U.S. to SI, multiply by	U.S. unit	SI unit	To convert SI to U.S., multiply by
29.574	fl oz	μL	3.3814×10^{-5}
29.5735	fl oz	mL	0.0338
3.7854	gal	L	0.2642
16.3871	inch ³	cm ³	0.0610
0.4536	lb	kg	2.2046
28.3495	oz	g	0.0353

Sharma and Rana, 1999). Bacterial wilt has been reported for other members of the Zingiberaceae family, including *Alpinia* [*Alpinia* spp. (Hayward, 1994)], turmeric [*Curcuma longa* (Vellupillai, 1986)], galanga [*Kaempferia galanga* (He, 1986)], siam tulip (*Curcuma alismatifolia*), and mioga [*Zingiber mioga* (Tsuchiya et al., 2004, 2005)]. The pathogen was isolated from yellow ginger (*Hedychium flavescens*), white ginger (*Hedychium coronarium*), and kahili ginger plants growing at a single location on the island of Oahu in Hawaii (Aragaki and Quinon, 1965).

A ginger strain of *Rs* originally isolated from edible ginger has been used in a biocontrol program to reduce populations of kahili ginger, an invasive Zingiberaceae species in the tropical forests of Hawaii. The ginger strain was reportedly specific for kahili ginger and nonpathogenic to important ornamental gingers and common plant species in Hawaii (Anderson and Gardner, 1999). Based on this premise, the pathogen was introduced into Hawaiian forests, including Hawaii Volcanoes National Park on the island of Hawaii (Anderson and Gardner, 1996, 1999). However, other studies indicated ornamental ginger species were susceptible to the ginger strain (Paret et al., 2006). Thus, further studies were needed to assess the host range using a representative set of the *Rs* strains isolated from edible ginger and kahili ginger.

Survival of *Rs* races 1, 2, and 3 in water, soil-sand mixtures, and roots of symptomless solanaceous hosts has been documented (Elphinstone et al., 1998; Granada and Sequeira, 1983; Hayward, 1991), but there is a lack of information on survival of race 4 ginger strains in soil or potting media that are commonly used in Hawaii to produce rhizomes in bag culture (Hepperly et al., 2004). Because the industry has increased the use of potting media for production of ornamental gingers (Criley et al., 2005; Kuehny et al., 2005a, b), survival of *Rs* race 4 populations in the potting medium may be a factor in disease spread.

The objective of the current study was to assess the pathogenicity of *Rs* race 4 strains on plants belonging to the Zingiberaceae and Costaceae families, and to determine the

ability of the pathogen to survive in a potting medium commonly used for ginger culture in Hawaii.

Materials and methods

PATHOGENICITY AND HOST RANGE STUDIES. *Ralstonia solanacearum* strains used for the experiments (A4515, A5192, and A3450) were selected from a larger collection of 55 well-characterized ginger and tomato strains to represent the genetic diversity observed in the local population (Yu et al., 2003). For host range studies we selected two edible ginger strains (A4515 and A5192; isolated from farms on the island of Hawaii), one kahili ginger strain (A4679; isolated from the island of Hawaii), and three tomato strains (A5370, A3450, and A5345; isolated from Hawaii, Trinidad, and Florida respectively). A nonpathogenic strain of *Enterobacter cloacae* (*Ecl*) A5149, an endophyte of the ginger rhizome (Nishijima et al., 2004), isolated from the island of Hawaii was used as a negative control. Bacterial strains were streaked on a modified tetrazolium chloride (TZC) medium (Norman and Alvarez, 1989) and incubated for 48 to 72 h. One to two colonies of *Rs* were transferred to 3.0 mL distilled water (dH₂O), vortexed, and adjusted spectrophotometrically to $A_{600\text{nm}} = 0.1$, which corresponds to 10^8 cfu/mL. Seeds of important ornamental gingers such as red ginger, pink ginger, white ginger, red ginger lily (*Hedychium coccineum*), siam tulip, white turmeric (*Curcuma zedoaria*), Globba (*Globba* spp.), yellow ginger, shampoo ginger, kahili ginger, beehive ginger (*Zingiber spectabile*), torch ginger (*Etlingera elatior*), and edible ginger belonging to the Zingiberaceae family; and spiral ginger (*Costus barbatus*), belonging to the Costaceae family, were collected from the Waimanalo Farm and Lyon Arboretum (University of Hawaii at Manoa, Manoa, HI) and planted in plastic pots (2 L) filled with potting medium (Sunshine Mix 4 Aggregate Plus; Sun Gro Horticulture Canada Ltd, BC, Canada). Two- to three-month-old plants were inoculated into the stem using a syringe to deliver 1.0 mL of the inoculum for each strain tested. Wilt symptoms including flagging and yellowing of leaves were recorded for 5 to 21 d after inoculation (DAI).

The *Ecl* strain (A5149), used as a negative control, was inoculated at the same cell density as *Rs* strains. All strains were tested on one to nine plants per species depending on plant availability. The pathogenicity assessment was based on the percentage of severely wilted plants up to 21 DAI.

SIMULATION OF NATURAL INFECTION BY *RALSTONIA SOLANACEARUM* RACE 4. The ability of the pathogen to enter non-wounded roots was evaluated by a method to simulate natural infection. In our preliminary studies we noted that both ginger strains and kahili ginger strain wilted edible ginger and ornamental gingers 7 to 10 DAI (data not shown), and hence we selected *Rs* kahili ginger strain A4679 for our experiment. Twenty-five milliliters of the bacterial suspension containing $\approx 10^8$ cfu/mL of the strain was poured onto the base of 2- to 3-month-old disease-free red ginger, pink ginger, white ginger, red ginger lily, yellow ginger, shampoo ginger, kahili ginger, beehive ginger, spiral ginger, shell ginger, and edible gingers (planted in 2-L plastic pots filled with Sunshine Mix 4 Aggregate Plus potting medium). Three plants of each ginger species were inoculated and monitored for wilting symptoms up to 3 months. Pathogen identity in infected tissue samples was confirmed using samples of *Rs*-specific immunostrips (Agdia Inc., Elkhart, IN). A 1-g plant sample ($\approx 1 \text{ cm}^3$), was excised from the collar region of the infected plant, surface sterilized with 0.6% sodium hypochlorite (NaOCl) for 2 min, rinsed with dH₂O three times, and ground with a mortar and pestle in 1 mL dH₂O. One hundred microliters of BEB1 buffer (Agdia Inc.) was mixed in a microfuge tube with 100 μL of the sample extract and tested with the immunostrip assay.

SURVIVAL OF *RALSTONIA SOLANACEARUM* RACE 4 IN POTTING MEDIUM. The potting medium used for bag culture (Hepperly et al., 2004) was inoculated by pouring 25 mL of an aqueous suspension containing 10^8 cfu/mL of ginger strain A4515 into each of four pots (2 L) containing single 5-month-old edible ginger plants. The plants were watered daily with 200 mL water for 240 d. Each day, 50 mL effluent water was collected and vortexed;

Table 1. Pathogenicity of *Ralstonia solanacearum* (Rs) on various species in Zingiberaceae and Costaceae.

Bacterium	Strain no. ^z	Host of origin	Origin	Ginger species tested ^y (no. plants wilted/no. plants tested)													
				1	2	3	4	5	6	7	8	9	10	11	12	13	14
Rs race 4	A4515	Edible ginger	HI	4/4	2/4	3/3	3/3	1/1	0/1	3/3	3/3	3/3	3/3	1/1	0/1	3/3	6/6
	A5192	Edible ginger	HI	3/4	2/4	5/5	3/3	2/2	1/1	3/3	3/3	3/3	2/3	1/1	0/1	3/3	4/4
	A4679	Kahili ginger	HI	3/3	3/3	6/6	5/5	NT	NT	NT	5/5	4/4	4/4	3/3	NT	3/3	6/9
Rs race 1	A5370	Tomato	HI	4/7	3/3	2/3	3/3	NT	NT	NT	2/3	3/3	1/2	1/1	0/1	NT	0/1
Rs race 1	A3450	Tomato	TR	0/4	NT	0/3	0/2	0/1	0/1	0/1	0/2	0/2	0/1	NT	NT	0/4	0/5
	A5345	Tomato	FL	0/3	0/3	0/3	0/3	NT	NT	NT	NT	NT	NT	NT	NT	NT	0/1
Ecl	A5149	Edible ginger	HI	0/3	0/3	0/4	0/3	0/1	0/1	0/1	0/2	0/2	0/1	0/1	0/1	(0/4)	0/3

^aProviders of bacterial strains: A4515, E.E. Trujillo; A4679, D.E. Gardner; A3450, D. Cook; A5345, T.M. Momoi; and A5192, A5370, and A5149 (our collection).

^bZingiberaceae: 1, red ginger; 2, pink ginger; 3, white ginger; 4, red ginger lily; 5, siam tulip; 6, white turmeric; 7, globba; 8, yellow ginger; 9, shampoo ginger; 10, kahili ginger; 11, beehive ginger; 12, torch ginger; 13, edible ginger.

Costaceae: 14, spiral ginger.
The results were recorded 21 d after inoculation (DAI) of the stem of 2- to 3-month-old plants with *Ralstonia solanacearum* strains representing race 4 and race 1. The *Enterobacter cloacae* (Ec1) strain A5149 is the negative control.
FL, Florida; HI, Hawaii; NT, not tested; Rs, *Ralstonia solanacearum*; TR, Trinidad.

a 1-mL subsample was taken to initiate a 10-fold dilution series (10^{-1} to 10^{-7}); 100 μ L of each dilution was plated onto modified SMSA medium (Engelbrecht, 1994) to assess the viable Rs population. The same method was used to assess the bacterial population in the potting medium. A 1-g soil sample was vortexed for 10 s in 10 mL dH₂O, and allowed to settle. One milliliter of the supernatant was diluted to enumerate colony counts by the previously mentioned methods. The effluent water and potting medium samples from

this experiment were analyzed until 240 DAI.

Three methods of inoculation—pouring inoculum into plant-free potting medium, stem inoculation, and rhizome inoculation—were compared in another experiment to determine the effect of the bacterial inoculation method (hence, plant part colonized by the pathogen) and subsequent release of Rs into the potting medium and effluent water. For the first inoculation method, 25 mL of an aqueous suspension containing 10^8 cfu/mL of the ginger

Fig. 1. Symptoms on six ginger species 5 to 21 d after inoculation (DAI) with *Ralstonia solanacearum* strains (left to right) A4515, A5192, A5370, and *Enterobacter cloacae* strain A5149 (negative control). Symptoms noted are yellowing, flagging, and wilting of leaves. From top: row 1, white ginger; row 2, red ginger lily; row 3, yellow ginger; row 4, kahili ginger; row 5, beehive ginger; row 6, shampoo ginger.

strain of *Rs* A4515 was poured into pots (2 L) containing potting medium but no plants. For the second method, 1.0 mL *Rs* was inoculated into each stem (three stems inoculated per plant) with a syringe. For the third method, the rhizome was wounded with a scalpel, followed by pouring 27 mL inoculum into the base of the plant. Two pots (2L) were tested for each method; inoculated plants were 5 months old. The pots were watered daily with 200 mL water for 180 d. Fifty milliliters of effluent water was collected and vortexed each day. A 1-mL subsample was then taken to initiate a 10-fold dilution series (10^{-1} to 10^{-7}); 100 μ L of each dilution was plated onto modified SMSA medium to assess the viable *Rs* population. We also enumerated the bacterial population in potting medium by removing a 1-g soil sample from the pot, vortexing for 10 s in 10 mL dH₂O, and allowing soil particles to settle for 5 min. One milliliter of the supernatant was then diluted to enumerate viable colonies.

Results and discussion

PATHOGENICITY AND HOST RANGE STUDIES. The race 4 ginger strains affected nearly all ornamental ginger species tested (Table 1). Symptoms ranged from flagging or wilting to plant death. Although the majority of the plants wilted within 10 DAI, the final pathogenicity assessment was recorded 21 DAI, at which time all affected plants were severely wilted or dead. The *Ecl* negative control did not wilt any of the plants tested (Fig. 1). Of the three race 1 tomato strains tested, strain A5370 (isolated from tomato in Hawaii) wilted nearly all ginger species, whereas strains A3450 and A5345 (isolated from tomato in Trinidad and Florida respectively) did not wilt any of the ginger species. Most of the infected ginger plants showed moderate to complete wilting of leaves, and none of the ginger species were resistant to the *Rs* race 4 strains, with the possible exception of torch ginger (Table 1), which showed no symptoms. However, only a few plants of this species were available for testing, so results are inconclusive. In contrast to previously published data (Anderson and Gardner, 1999), our results show that the race 4 strains are pathogenic to many ornamental gingers.

Fig. 2. Number of days for severe wilting (100% dead plants) of Zingiberaceae and Costaceae plants in tests to simulate natural infection by pouring *Ralstonia solanacearum* race 4 kahili ginger strain A4679 into the potting medium at the base of the plant. RG, red ginger; PG, pink ginger; WG, white ginger; RGL, red ginger lily; YG, yellow ginger; SG, shampoo ginger; KG, kahili ginger; BG, beehive ginger; SpG, spiral ginger; ShG, shell ginger; EG, edible ginger. The error bars represent SD from the average of three plants.

Fig. 3. Recovery of *Ralstonia solanacearum* (*Rs*) race 4 from effluent water of pots containing infected edible ginger. The edible ginger plants were rhizome wounded and poured with *Rs* strain A4515, and effluent water from the pots was routinely collected and analyzed to assess the bacterial population for a period of 240 d. Viable cells are reported as log colony-forming units per milliliter (1 cfu/mL = 29.5735 cfu/fl oz). Each point is the average of four samples and the error bars indicate SD from the mean. The strain tested is *Rs* race 4, A4515.

Fig. 4. Survival of *Ralstonia solanacearum* (*Rs*) race 4 in potting medium containing infected edible ginger. The edible ginger plants were rhizome wounded and poured with *Rs* strain A4515, and potting medium from these pots was collected and analyzed to assess the bacterial population for a period of 240 d. Viable cells are reported as log colony-forming units per milliliter (1 cfu/mL = 29.5735 cfu/fl oz). Each point is the average of four samples and the error bars indicate SD from the mean. The strain tested is *Rs* race 4, A4515.

Spread of *Rs* from infected siam tulip to the edible ginger and, subsequently, to mioga fields in the Kochi prefecture of Japan was well documented from 1995 to 1999 (Tsuchiya et al., 2005). Our findings that the *Rs* race 4 strain from kahili ginger is pathogenic to numerous plant species belonging to the Zingiberaceae and Costaceae families thus has broad implications in Hawaii because of the reported use of this strain as a biocontrol agent for invasive kahili ginger in forest reserves.

SIMULATION OF NATURAL INFECTION BY *RALSTONIA SOLANACEARUM* RACE 4. The kahili ginger strain of *Rs* (A4679) wilted all 11 ginger species tested when plants were inoculated without wounding (Fig. 2). Shampoo ginger, beehive ginger, spiral ginger, and kahili ginger were highly susceptible and died within 38 d. Shampoo ginger was the most susceptible and wilted in 26 d, whereas pink ginger was the least susceptible, wilting in 76 d. The presence of the pathogen in stem sections of each species that showed wilting was confirmed by the immunostrip assay. The observation that the pathogen invaded the host even in the absence of a stem or root wound confirms its ability to enter through natural openings. This suggests that the pathogen could spread naturally from the inoculated forest plants to edible ginger and ornamental ginger production areas.

LONG-TERM SURVIVAL OF *RALSTONIA SOLANACEARUM* RACE 4 IN POTTING MEDIUM. *Ralstonia solanacearum* race 4 survived for 180 DAI and was detected at low levels for 150 to 180 DAI in effluent water (Fig. 3). The pathogen was also detected in potting medium 180 DAI (Fig. 4).

In the subsequent experiment, which tested the survival of pathogen after three inoculation methods, the population of the pathogen was no longer recovered from effluent water of plant-free potting medium at 30 d, in comparison with recovery of high populations from effluent water of pots containing stem- and rhizome-inoculated plants (Fig. 5). High populations of *Rs* were released from infected plants during the early stages of disease development. The pathogen was recovered from effluent water up to 150 DAI with stem- and rhizome-inoculated plants, and 60 DAI

from effluent water of plant-free potting medium (Fig. 6). However when potting medium was sampled instead of effluent water, the pathogen survived with stem- or rhizome-inoculated plants for 150 DAI and for 120 DAI in plant-free potting medium (Fig. 7). High populations of *Rs* were noted when potting medium samples

were analyzed instead of effluent water from these pots for all three methods of inoculation. The survival of the pathogen in the absence of host plant debris for 4 months is of particular concern to local growers, because it is an indication that the pathogen may persist in an ecosystem even after infected plants are removed.

Fig. 5. Recovery of *Ralstonia solanacearum* (*Rs*) 1 to 30 d after inoculation from effluent water after inoculation with three methods: plant-free potting medium (▲), stem inoculation (◆), and rhizome inoculation (■). Plant-free potting medium represents pots filled with potting media and with no plants that were inoculated with *Rs*, and this represents the free-living bacteria. Stem inoculation represents pots with edible ginger, inoculated on the stem with *Rs*. Rhizome inoculation represents pots with edible ginger, and the rhizome was wounded, followed by pouring *Rs* inoculum. Viable cells are reported as log colony-forming units per milliliter (1 cfu/mL = 29.5735 cfu/fl oz). Each point is the average of two samples per treatment and the error bars indicate SD from the mean. The strain tested is *Rs* race 4, A4515.

Fig. 6. Recovery of *Ralstonia solanacearum* (*Rs*) for 180 d from effluent water after inoculation with three methods: plant-free potting medium (▲), stem inoculation (◆), and rhizome inoculation (■). Plant-free potting medium represents pots filled with potting media and with no plants that were inoculated with *Rs*; this represents the free-living bacteria. Stem inoculation represents pots with edible ginger, inoculated on the stem with *Rs*. Rhizome inoculation represents pots with edible ginger, and the rhizome was wounded, followed by pouring *Rs* inoculum. Viable cells are reported as log colony-forming units per milliliter (1 cfu/mL = 29.5735 cfu/fl oz). Each point is the average of two samples per treatment and the error bars indicate SD from the mean. The strain tested is *Rs* race 4, A4515.

Fig. 7. Survival of *Ralstonia solanacearum* (*Rs*) race 4 in potting medium for 180 d after inoculation with three methods: plant-free potting medium (▲), stem inoculation (◆), and rhizome inoculation (■). Plant-free potting medium represents pots filled with potting media and with no plants that were inoculated with *Rs*; this represents the free-living bacteria. Stem inoculation represents pots with edible ginger, inoculated on the stem with *Rs*. Rhizome inoculation represents pots with edible ginger, and the rhizome was wounded, followed by pouring *Rs* inoculum. Viable cells are reported as log colony-forming units per milliliter (1 cfu/mL = 29.5735 cfu/fl oz). Each point is the average of two samples per treatment and the error bars indicate SD from the mean. The strain tested is *Rs* race 4, A4515.

Pathogen-free edible ginger seed rhizomes are currently produced in potting medium in Hawaii (Hepperly et al., 2004), and many ornamental flower growers use this potting medium for production. The ability of *Rs* to survive in this medium after it is introduced presents risks to both the edible ginger and floriculture ginger industries. Further studies of long-term survival of *Rs* in volcanic soil and ginger field soil are in progress.

Conclusions

The results of pathogenicity tests, simulation of natural infection, and long-term survival studies indicate that *R. solanacearum* race 4 can affect negatively both the edible ginger and floriculture ginger industries in Hawaii. Use of *Rs* as a biocontrol agent against an invasive ginger species is a concern to local agriculturalists, and a thorough evaluation of its spread, and survival on weeds and in watercourses is needed before any application in forest reserves.

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