

RESEARCH ARTICLE

## Systemic resistance induced by *Trichoderma harzianum* RU01 against *Uromyces appendiculatus* on *Phaseolus vulgaris*

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**Abstract:** *Trichoderma* spp. are effective biocontrol agents for a number of soil-borne plant pathogens. Some isolates are also known for their ability to induce systemic resistance in plants against different foliar pathogens. Induction of systemic resistance against *Uromyces appendiculatus*, the causal agent of bean rust, by *Trichoderma* spp. has not been reported. *T. harzianum* RU01 consistently triggered a significant ( $P=0.05$ ) degree of protection against rust in bean (*Phaseolus vulgaris* cv. Keppetipola Nill) under greenhouse conditions. Control efficacy was similar to that of rhizobacterium, *Pseudomonas aeruginosa* KMPCH, a test strain included in this study, which previously demonstrated induction of systemic resistance in bean. Given the spatial separation of challenging pathogen and biocontrol agent, this effect can be attributed to the induction of systemic resistance by *T. harzianum* RU01.

**Keywords:** Bean rust, biological control, biotrophs, induced resistance, *Trichoderma* spp.

### INTRODUCTION

Rust caused by *Uromyces appendiculatus* Pers., is one of the major diseases of bean (*Phaseolus vulgaris* L.) in Sri Lanka and many other parts of the world. Severe crop loss can result when epidemics occur<sup>1</sup>. Control of bean rust is achieved using resistant cultivars<sup>2</sup> and application of certain fungicides<sup>3</sup>. However, concerns about public health and the development of resistant strains of pathogens have increased the search for alternative methods of crop protection. At the same time the cost involved in chemical pesticides is a major concern for farmers in developing countries.

There have only been few reports describing the control of bean rust pathogen by means other than chemical fungicides. Among the few examples bacterial antagonist *Bacillus subtilis*<sup>1</sup>, *Pantoea agglomerans* and

*Stenotrophomonas maltophilia* amended with colloidal chitin<sup>4</sup> demonstrated successful control of bean rust under greenhouse and field conditions. The use of synthetic plant resistance inducers against *U. appendiculatus* has also been reported<sup>5,6</sup>.

Contact with pathogenic and non-pathogenic micro-organisms triggers a wide range of induced defense mechanisms in plants that protect them against invasion. One such response is known as systemic acquired resistance (SAR)<sup>7</sup>. Another form is associated with the colonization of plant roots by certain plant growth promoting rhizobacteria (PGPR), referred to as induced systemic resistance (ISR)<sup>8</sup>. This phenomenon has been demonstrated in many host plants including bean<sup>9</sup> against different plant pathogens. The ability of selected bacterial strains to induce synthetic resistance in bean against bean rust has been recently reported (Abeysinghe, *in press*)<sup>10</sup>. Apart from the PGPR and synthetic ISR inducers, certain strains of *Trichoderma* isolates have also been reported as ISR inducers in different plants including bean<sup>11-15</sup>. However ISR induced by *Trichoderma* spp. in bean against *U. appendiculatus* has not been reported.

Therefore, the main objective of the present study was to determine whether *Trichoderma harzianum* RU01 can induce systemic resistance in bean against the bean rust pathogen *U. appendiculatus* under greenhouse conditions.

### METHODS AND MATERIALS

*The biocontrol agents:* *Trichoderma* isolates were isolated from soil samples obtained from commercial agricultural fields at Angunukolapalasse, in the Southern

Province of Sri Lanka by using the dilution Petri plate technique on *Trichoderma* Selective Medium (TSM)<sup>16</sup>. Isolates were maintained on Potato Dextrose Agar (PDA). Identification was performed by using colony characteristics described by Bissett. *T. harzianum* RU01 was selected for further studies because of its ISR inducing ability against *U. appendiculatus* in preliminary experiments. *Pseudomonas aeruginosa* KMPCH, was included in the present study as a positive ISR inducing strain in bean as described by De Mayer *et al.*<sup>11</sup>.

*Inoculum production and seed inoculation with T. harzianum RU01:* For seed inoculation, *T. harzianum* RU01 was grown on PDA in Petri plates for 7 d at room temperature under dark to allow profuse sporulation. Sterile distilled water (SDW) was added to each plate and a conidia suspension was obtained by scraping the colony surface with a sterile spatula and then filtering through cheese cloth. The conidia suspension was adjusted to 10<sup>8</sup> conidia/mL. Tween-20 (0.01% v/v) was added as a wetting agent. Viability of the spores was checked by plating serially diluted conidia suspension on PDA plates. Bean seeds (*Phaseolus vulgaris* cv. Keppetipola Nill) were soaked for 5 min in the conidial suspension and subsequently planted in a soil amended with 10<sup>8</sup> conidia/g soil. For this the conidia suspension was prepared as described above and thoroughly mixed with the soil (clay:sand:compost) (1:1:1) v/v. *P. aeruginosa* KMPCH was used as described in De Mayer *et al.*<sup>11</sup>.

*Plant growing conditions:* Bean plants (*P. vulgaris* cv. Keppetipola Nill), a susceptible cultivar to *U. appendiculatus*, were grown in 15 cm diameter plastic pots (4 seeds/pot and thinned to 2 uniform seedlings/pot after 1 wk) containing soil (clay:sand:compost) (1:1:1) v/v maintained at 28°C/22°C ± 2 day/night temperatures in the greenhouse at the Department of Botany, University of Ruhuna. Ten pots were included for one treatment. Plants were fed weekly with an appropriate liquid fertilizer and used for experiments when 3 wks old. Ten milliliters of 1 mM salicylic acid (SA) was added as a soil drench directly to the stem base at day 10 and 15. The other plants received the same amount of water.

*Pathogen inoculation and scoring of disease symptoms:* Bean leaves infected with rust bearing matured pustules were collected from the bean fields located at Bandarawela and brought to the laboratory. Collection was essentially done from the same location throughout the experiment in order to prevent combination of different races of the pathogen. The pustules were scraped into sterile distilled water amended with Tween 20 at 20 µL/L and made into the urediniospore suspension (10<sup>5</sup> spores/mL). The abaxial part of each of the primary leaves was inoculated

with this suspension by using a spore sprayer (atomizer) to cover the leaf surface homogeneously and each pot was covered with a polyethylene bag immediately. For the initial 24 h after the inoculation, these pots were kept in a dark cool place and then transferred to a shaded area to prevent direct light. 48 h after inoculation, the mouths of the bags were opened but the pots were kept inside the bags in order to maintain high humidity around the leaves. Ten days after incubation, the leaves were scored for rust incidence by counting the number of pustules within 3 randomly selected 4 cm<sup>2</sup> areas per leaf. Occurrence of uredosporic pustules was counted as disease incidence, whereas small necrotic spots without uredospores were considered immune.

*Plant colonization assay:* In order to exclude the possibility of direct antagonism by the bacterial and *T. harzianum* RU01 isolates which can also colonize leaves, stems, and cotyledons of bean plants were checked for colonization. Plants were randomly selected from each treatment representation for each block and 1.0 g of primary leaf, roots and stem base were separately macerated in 10 mL of SDW. The serially diluted extract (100 mL) was plated on King's B plates for bacteria<sup>11</sup> and *Trichoderma* selective medium<sup>16</sup> for *T. harzianum* RU01 respectively. Colony counts, if any, were made after 48 h of incubation. The experiment was performed three times for each plant.

*Plant dry weight:* Mean plant dry weight was determined at the end of the experiment. The plants (one plant representing each block per treatment, not inoculated with the pathogen) were uprooted with care and the soil clumps removed by rinsing under tap water. The plants were dried at 80°C until a constant dry weight was reached.

*Statistical analysis:* Treatments were arranged in a randomized complete block and the pot experiments were repeated 3 times. Results were analysed by ANOVA using JMP software. The significance of effect of biocontrol treatments was determined by the magnitude of the *F* value (*P* = 0.05). When a significant *F* test was obtained for treatments, means were compared by Fisher's protected least significant difference (LSD).

## RESULTS AND DISCUSSION

For several decades *Trichoderma* spp. have been known for their ability to act as biological control agents against plant pathogens<sup>16</sup>. Some strains inhibit or eradicate propagules of plant pathogens in the soil or on roots of plants through antagonism and mycoparasitism. Some *Trichoderma* strains colonize roots and exert their effect

on foliar pathogens through systemic action<sup>15</sup>. Results of the present investigation revealed the potential of *T. harzianum* RU01 to reduce severity of rust infection on bean caused by *U. appendiculatus*. This is the first report of induction of systemic resistance against bean rust by a *Trichoderma* strain.

Uredinia pustules appeared on primary leaves 7 d after

**Table 1:** Systemic resistance induced by different treatments against bean rust in bean cv. Keppetipola Nill under greenhouse conditions

Treatment	Trial I	Trial II	Trial III
	Mean number of uredino pustules per plant*		
Untreated control	46 a <sup>‡</sup>	58 a <sup>‡</sup>	50 a <sup>‡</sup>
<i>T. harzianum</i> RU01	22 b	26 b	22 b
<i>P. aeruginosa</i> KMPCH	25 b	28 b	24 b
1.0 mM Salicylic acid**	12 c	16 c	10 c

\* Three week old primary leaves were inoculated with an uredino spore suspension ( $10^5$  conidia/mL) and incubated for 48 h under humid conditions and transferred to the controlled environment condition in the greenhouse. The number of uredosporic pustules emerging from the epidermis 10 d after inoculation was counted as the disease incidence. For each trial, data were collected from 5 plants and 3 replications were included per treatment.

\*\*1.0 mM salicylic acid was applied as a soil drench (10 mL) at day 10 and 15, directly to the stem base of the bean plants.

<sup>‡</sup> Mean values in the same column followed by the same letter are not statistically significant according to Fisher's least significant test ( $p = 0.05$ ).

challenge inoculation with *U. appendiculatus*, a few days earlier than what was reported in previous studies<sup>5</sup>. This difference could be due to the high humidity maintained around the leaves during the first 48 h in this study. The number of pustules was always easily countable suggesting that the simple experimental setup can be used for assessing pathogenicity of bean rust pathogen as indicated in this study.

*T. harzianum* RU01 and *P. aeruginosa* KMPCH evaluated in the greenhouse, significantly ( $P = 0.05$ ) reduced disease incidence consistently as compared to the untreated control plants (Table 1). However, resistance induced by the synthetic inducer salicylic acid (SA) conferred the highest resistance level compared to the biological inducers (Table 1). Previous studies<sup>5,6,18</sup> showed the ability of synthetic SAR inducers to protect bean plants from *U. appendiculatus* and the current study is in accordance with these reports. Moreover, the sizes

of rust pustules in SA treated plants were smaller than that in controls and the plants treated with biocontrol agents (data not shown). This is in agreement with a previous study<sup>5</sup> where 2, 6-dichloro-isonicotinic acid was used as a systemic resistance inducer against bean rust. SA is known as a putative signal molecule for defense responses in plants against plant pathogens<sup>19</sup>. Therefore, SA itself or any other signal generated by SA moves through the plant, and sensitizes bean leaves either to react faster upon pathogen invasion or to switch on defense reactions quickly. This may suggest that two or more different mechanisms are involved in systemic resistance induced by biocontrol agents<sup>20</sup>.

In order for the protective effect induced by *T. harzianum* RU01 in bean to be due to systemic activity, it is imperative to the objective of this work that the inducer remains spatially separated from the challenging pathogen in the host plant<sup>8</sup>. Based on isolations on the *Trichoderma* selective medium, *T. harzianum* RU01 was consistently recovered from roots of bean plants grown from seeds inoculated with and soil amended with the biocontrol agent but not from primary leaves (Table 2). Thus, spatial separation between the pathogen and the biocontrol agent was maintained, suggesting that the observed disease-resistance effect induced in bean against bean rust was due to systemic activity induced by *T. harzianum* RU01 and not a result of direct antagonism. The reference biocontrol bacterium included in this study, *P. aeruginosa* KMPCH, induced systemic resistance against bean rust in the cultivar used

**Table 2:** Population of biocontrol agents on primary leaves, stem base and roots of bean plants

Treatment <sup>a</sup>	Leaves	Stem base	Roots
	Mean colonization (log CFU/g) <sup>b</sup>		
Control	ND <sup>c</sup>	ND <sup>c</sup>	ND <sup>c</sup>
<i>T. harzianum</i> RU01	ND <sup>c</sup>	1.47	7.28
<i>P. aeruginosa</i> KMPCH	ND <sup>c</sup>	1.32	5.28

<sup>a</sup> Biocontrol agents were applied to the seed and amended to the soil as a suspension before being planted. Set of plants were not inoculated with the pathogen and used for dry weights at the end of the experiment.

<sup>b</sup> Plants were randomly selected from and 1.0 g of primary leaf, roots and stem base separately macerated with 10 mL of SDW, and 100  $\mu$ L of the serially diluted extract was plated on King's B plates for bacteria according to De Mayer *et al.* and *Trichoderma* selective medium (Askew and Laing 1993) for *T. harzianum* RU01. Colony counts if any were made after 48 h of incubation. The experiment was performed three times for each plant.

<sup>c</sup> ND = not detected

in this study to the same extent induced by *T. harzianum* RU01. These findings further support our conclusion that the suppressive effect against bean rust by *T. harzianum* RU01 was due to systemic resistance.

In the present study we did not detect significant changes in plant growth (Table 3) and these findings agree with earlier reports on other *T. harzianum* isolates that systemic resistance was induced without affecting

**Table 3:** Dry weight of the bean plants treated with biocontrol agents

Treatment	Dry weight g/ plant*
Control	1.54 ± 0.22a <sup>1</sup>
<i>T. harzianum</i> RU01	1.84 ± 0.32a
<i>P. aeruginosa</i> KMPCH	1.24 ± 0.18a

\* Mean plant dry weight was determined at the end of the experiment. One plant for each block per treatment was not inoculated with the pathogen and used for dry weights. The plants were dried at 80°C until a constant dry weight was reached.

<sup>1</sup> Mean values in the same column followed by the same letter are not statistically significant according to Fisher's least significant test ( $p = 0.05$ ).

growth of the host plant<sup>15,21</sup>. Lack of an impact of *T. harzianum* RU01 on growth in the present study is possibly due to the short period used (5 wks). Therefore, it is not possible to make any concrete conclusion at the present time.

In conclusion, the findings strongly support the concept that *T. harzianum* RU01 actively induces systemic resistance against bean rust caused by *U. appendiculatus*. The nature of systemic resistance activated by *Trichoderma* spp. remains unclear<sup>22</sup>. Activation of defence-related molecules has been noted in roots and distant parts of colonized plants<sup>15</sup>. As mentioned in the introduction, several non-pathogenic root-colonizing bacteria and fungi, notably of the genus *Pseudomonas* and *Trichoderma* respectively are able to suppress pathogens by direct antagonism or by induction of systemic resistance in the plant extending protection to foliar pathogens. The transduction mechanisms involved in systemic resistance induced by these biocontrol agents are reported to be varied among the inducing agents<sup>20</sup>. Shores et al.<sup>14</sup> have shown that *T. asperellum* T203 induced systemic resistance is not an SA-dependent phenomenon but rather requires components of the jasmonic acid (JA) signaling pathway. In the case of rhizobacteria induced systemic resistance, it has been

shown that both SA-dependent and SA-independent pathways are involved depending on the inducing agent<sup>8</sup>. However, to the best of our knowledge SA-dependent systemic resistance induction by *Trichoderma* spp. has not been reported. Therefore, unraveling the possible mode of action behind the activation of systemic resistance in bean by *T. harzianum* RU01 is of paramount importance.

It is important to realize here that enhancement of resistance by *T. harzianum* RU01 is effective only to a limited extent ( $\leq 50\%$ ). Therefore, further means of protection will be required to maintain the protection at or below the economic threshold level due to the polycyclic nature of the pathogen under field conditions. Thus, the results indicate the necessity of combining several approaches along with biological control in order to get effective management of bean rust.

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