



RESEARCH ARTICLE

**REVISED** Evaluation of *Trichoderma* spp., *Pseudomonas fluorescens* and *Bacillus subtilis* for biological control of *Ralstonia* wilt of tomato

Shiva Yendyo <sup>1</sup>, Ramesh G.C. <sup>2</sup>, Binayak Raj Pandey <sup>2</sup>

<sup>1</sup>Kishan Call Center, Bharatpur-4, Chitwan, 44207, Nepal

<sup>2</sup>Department of Quality Control, Agricare Nepal Pvt. Ltd., Bharatpur-4, Chitwan, 44207, Nepal

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**Abstract**

**Background:** *Ralstonia* spp. is a major pathogenic microbe for tomato, which invades the roots of diverse plant hosts and colonizes xylem vessels causing wilt, especially in tropical, subtropical and warm-temperate regions. *Ralstonia* spp. produces several virulence factors helping it to invade the plant's natural defense mechanism. Native isolates of *Trichoderma* spp., *Pseudomonas fluorescens* and *Bacillus subtilis* can be used as biocontrol agents to control the bacterial wilt and combined application of these beneficial microbes can give better results.

**Methods:** Bacterial wilt infection in the field was identified by field experts and the infected plant part was used to isolate *Ralstonia* spp. in CPG media and was positively identified. Subsequently, the efficacy of the biocontrol agents was tested and documented using agar well diffusion technique and digital microscopy. 2ml of the microbial concentrate (10<sup>9</sup> cells/ml) was mixed in one liter of water and was applied in the plant root at the rate of 100 ml per plant as a treatment method.

**Results:** It was observed that the isolated *Trichoderma* spp. AA2 and *Pseudomonas fluorescens* PFS were most potent in inhibiting the growth of *Ralstonia* spp., showing ZOI 20.67 mm and 22.33 mm, respectively. Digital microscopy showed distinct inhibitory effect on the growth and survival of *Ralstonia* spp. The results from the field data indicated that *Trichoderma* spp. and *Pseudomonas fluorescens* alone were able to prevent 92% and 96% of the infection and combination of both were more effective, preventing 97% of infection. Chemical control methods prevented 94% of infection. *Bacillus subtilis* could only prevent 84 % of the infection.

**Conclusions:** Antagonistic effect against *Ralstonia* spp. shown by native isolates of *Trichoderma* spp. and *P. fluorescens* manifested the promising potential as biocontrol agents. Combined application gave better results. Results shown by *Bacillus subtilis* were not significant.

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- 1 **Teresa A. Coutinho**, University of Pretoria, South Africa
- 2 **Dhurva P. Gauchan**, Kathmandu University, Nepal

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**Corresponding author:** Binayak Raj Pandey ([binayakrp@gmail.com](mailto:binayakrp@gmail.com))

**Author roles:** **Yendyo S:** Data Curation, Funding Acquisition, Investigation, Methodology, Resources, Validation; **G.C. R:** Data Curation, Formal Analysis, Investigation, Resources, Validation; **Pandey BR:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Resources, Software, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

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**REVISED Amendments from Version 2**

Minute changes have been made in the Results and Discussion sections as per referee input in version 2.

See referee reports

**Author endorsement**

Dr. Sushil Thapa confirms that the author has an appropriate level of expertise to conduct this research, and confirms that the submission is of an acceptable scientific standard. Dr. Sushil Thapa declares he has no competing interests. Affiliation: Texas A&M AgriLife Research, Amarillo, TX 79106, USA.

**Introduction**

Bacterial wilt caused by *Ralstonia* spp. is a major pathogenic microbe for tomatoes (*Solanum lycopersicum* L). It invades the roots of diverse plant hosts from the soil and aggressively colonizes the xylem vessels, causing bacterial wilt disease<sup>1-3</sup>. It is a devastating plant disease most commonly found in tropical, subtropical and warm-temperate regions<sup>4,5</sup>. *Ralstonia* spp. produces several known virulence factors, including extracellular polysaccharide (EPS), and a combination of plant cell wall-degrading enzymes, such as endoglucanase (EG) and polygalacturonase (PG). Mutants lacking EPS and EG shows reduced virulence<sup>6-8</sup>. The major virulence factors for this pathogen are plant cell wall-degrading polygalacturonases (PGs)<sup>9</sup>.

Various biocontrol agents are used to control the bacterial wilt caused by *Ralstonia* spp. *Trichoderma harzianum*<sup>10,11</sup>, *Trichoderma viride*<sup>12</sup>, *Trichoderma asperellum*<sup>13</sup>, *Trichoderma virens*<sup>14</sup>, *Pseudomonas fluorescens*<sup>15,16</sup> and *Bacillus subtilis*<sup>17</sup> are used as biocontrol agents to control bacterial wilt. Combination treatment methods using two or more of these agents are more effective in managing the disease than treatment using a single biocontrol agent<sup>10,18,19</sup>. Chemical bactericides and fungicides induce resistance in pathogens during long-term use, which ultimately makes the pathogen tolerant to these chemical applications<sup>20-23</sup>. Hence, there should be a focus on the use of biological methods to control plant disease.

This study focuses on evaluating the efficacy of different native isolates of *Trichoderma* species, *B. subtilis* and *P. fluorescens* against bacterial wilt disease caused by the pathogen *Ralstonia* spp. in the tomato plant. The study hypothesizes that the native isolates of *Trichoderma* spp., *B. subtilis* and *P. fluorescens* can be used as bioantagonistic agents to control bacterial wilt (*Ralstonia* spp.) of tomato. This study tries to establish the hypothesis by the microscopic examinations, agar well diffusion technique and field trials in infected tomato plots, by calculating their efficacy and comparing them with chemical methods of treatment.

**Methods****Selection of the study plot**

The sample collection and field trials were done at Agro Narayani Farm, Sukranagar, Chitwan District, Nepal (Latitude: 27.582016 and Longitude: 84.272259) where the bacterial wilt infection

was recorded in the previous harvest (done 3 months before the test). Also, *Ralstonia* spp. were observed in the Casamino acid-Peptide-Glucose (CPG) Agar plates from the soil samples. The tests in the field were conducted from 11 March 2017 to 8 July 2017 for 120 days in new transplants. At the time of transplant, compost fertilizer at the rate 2.94 kg/m<sup>2</sup>, urea at the rate of 23.59 gm/m<sup>2</sup>, potash at the rate of 29.49 gm/m<sup>2</sup>, DAP 19.66 gm/m<sup>2</sup>, borax at the rate 1.97 gm/m<sup>2</sup> and zinc at the rate 1.97 gm/m<sup>2</sup> were applied. At 45 days and 90 days of transplant NPK 20:20:20 was applied at the rate 9.83/m<sup>2</sup>. The spacing of the plant was 50 cm in double row system of 50 × 50 cm. The plot size was 50 m<sup>2</sup> and total of 8 plots were used having 100 plants per plot. Weather data were also collected from online resources as a reference.

**Identification of bacterial wilt infection in the field**

Physical symptoms, such as wilting of young leaves, discolored tissue at the dissected part of the stem base, and white, slimy ooze when the dissected part of the plant was kept in the glass of water, were used for the identification of infected plants<sup>24</sup>.

**Isolation and identification of *Ralstonia* spp.**

Bacterial wilt infection in tomato plants (*Solanum lycopersicum* L. var. Manisha) grown in Chitwan, Nepal, was positively identified by S. Yendyo of Kishan Call Center (KCC). Six whole plants were brought to the Quality Control laboratory of Agricare Nepal Pvt. Ltd. in a sterile bag for the isolation of the pathogen.

*Ralstonia* spp. was isolated from dissected sections of the infected tomato plants on Casamino acid-Peptide-Glucose (CPG) Agar (casein hydrolysate 1 g/l, peptone 10 g/l, glucose 5g/l, agar 15g/l) which is preferred media for isolation of *Ralstonia* spp. The stems of the infected plant were washed three times with autoclaved distilled water and then blot dried. After drying the stem were washed with 80% ethanol solution, then 1% sodium hypochlorite (NaOCl) solution was applied for 2 minutes. Final washing was done with autoclaved distilled water three times. The xylem of 2–3 cm from the stem was dissected and the sap was rubbed in CPG medium and inoculated for 48h at 28°C. Identification was done on the basis of the morphology of the colony on CPG medium, Gram staining and microscopic examination<sup>25,26</sup>.

**Isolation of antagonists**

A total of 13 strains were used in this study (see Table 1). Six strains of *Trichoderma* spp., two strains of *Pseudomonas fluorescens* and one strain of *Bacillus subtilis* (GenBank Accession No. MG952584) were isolated from soils and root soils collected from different sites in Nepal. ATCC 13525 strain of *P. fluorescens* from Microbiologics, St. Cloud, MN 56303, France was used as the reference for isolated *Pseudomonas* species. *Trichoderma harzianum*, *Trichoderma virens* and *Trichoderma asperellum* strains were provided by Tamil Nadu Agricultural University (TNAU), Coimbatore, Andhra Pradesh, India, and were used as reference species for isolated *Trichoderma* spp for two purposes. First, the reference species were used to identify the isolated species through morphological and microscopic analysis and second, the reference species were used to compare the antagonistic effects exhibited by the native isolates. All the species were collected from the tropical region

**Table 1. Biocontrol agents used to analyze bio-efficacy against *Ralstonia* spp.**  
This includes the shortcode given to the isolates for identification in this study.

Name of strains and source	Source	Code given
<i>Trichoderma</i> spp.	<i>Parthenium hysterophorus</i> L. rhizoplane soil	AA2
<i>Trichoderma</i> spp.	<i>Cannabis sativa</i> L. rhizoplane soil	AG3
<i>Trichoderma</i> spp.	<i>Solanum viarum</i> Dunal rhizoplane soil	AKD
<i>Trichoderma</i> spp.	Agricare Nepal Pvt. Ltd. top soil	A5
<i>Trichoderma</i> spp.	<i>Brassica juncea</i> L. spp. rhizoplane soil	A9
<i>Trichoderma</i> spp.	Chitwan National Park top soil	A10
<i>Trichoderma harzianum</i>	TNAU, India	ATH
<i>Trichoderma virens</i>	TNAU, India	ATV
<i>Trichoderma asperellum</i>	TNAU, India	ATA
<i>Bacillus subtilis</i> (MG952584)	Bhaktapur top soil	BS
<i>Pseudomonas fluorescens</i>	<i>Parthenium hysterophorus</i> L. rhizoplane soil	PFS
<i>Pseudomonas fluorescens</i>	<i>Chenopodium album</i> L. rhizoplane soil	PFB
<i>Pseudomonas fluorescens</i>	ATCC 13525	PFA

except *Bacillus subtilis* which was collected from mid hill regions of Nepal. The reason behind this was that the site from where the samples were collected was unaffected by the wilt disease compared to nearby sites where the bacterial wilt was severely present. The testing of the soil samples from the unaffected site revealed the substantial presence of *Bacillus subtilis* which may have induced systemic resistance in the plants.

*Trichoderma* spp. were isolated by serial dilution method using TSM agar plate (K<sub>2</sub>HPO<sub>4</sub>: 0.9 g/l, MgSO<sub>4</sub>: 0.2 g/l, KCl: 0.15 g/l, NH<sub>4</sub>Cl: 1.05 g/l, Glucose 3 g/l, Rose Bengal: 0.15 g/l, agar 20 g/l, streptomycin: 100 mg/l, tetracycline: 50 mg/l)<sup>27</sup>. 1 gm of each soil samples were suspended in 9 ml of sterile distilled water and vortexed (Accumax India, New Delhi-110058, India) for 5 min. The soil suspension was then serially diluted to 10<sup>-3</sup> and 10<sup>-4</sup>. Pour plate technique was used by mixing 1 ml of the diluted soil suspension in 3 TSM agar plates for each sample and incubated at 25°C for 5 days. The strains were purified on TSM agar plates using sub-culture technique.

*Pseudomonas fluorescens* were isolated by serial dilution method using King's B agar (Peptone: 20g/l, K<sub>2</sub>HPO<sub>4</sub>: 1.5 g/l, MgSO<sub>4</sub>: 1.5 g/l, glycerol: 10 ml/l, agar: 20g/l)<sup>28</sup>. 1 gm of each soil samples were suspended in 9 ml of sterile distilled water and vortexed (Accumax India, New Delhi-110058, India) for 5 min. The soil suspension was then serially diluted to 10<sup>-6</sup>. Pour plate technique was used by mixing 1 ml of the diluted soil suspension in 3 King's B agar plates for each sample and incubated at 27°C for 48 h and the fluorescence was observed in UV light. The fluorescent strains were purified on King's B agar plates using sub-culture technique.

*Bacillus subtilis* was isolated by serial dilution method using Nutrient Agar (Peptic digest of animal tissue: 5 g/l, NaCl: 5 g/l, beef extract: 1.5 g/l, yeast extract: 1.5 g/l, agar: 20g/l)<sup>29</sup>.

1 gm of each soil samples were suspended in 9 ml of sterile distilled water and vortexed (Accumax India, New Delhi-110058, India) for 5 min. The soil suspension was then serially diluted to 10<sup>-6</sup>. Pour plate technique was used by mixing 1 ml of the diluted soil suspension in 3 NA plates for each sample and incubated at 27°C for 48 h. The strains were purified on NA agar plates by using sub-culture technique.

#### Evaluating the efficacy of antagonists against *Ralstonia* spp.

All thirteen isolates were screened against *Ralstonia* spp. by agar well diffusion technique<sup>30</sup>. *Ralstonia* spp. on CPG agar plates were transferred to the nutrient broth and shaken in a rotary shaker (Talboys, Henry Troemner, LLC, USA) at 100 rpm at 27°C for 24 h. Similarly, the TSM, King' B and NB were prepared for all *Trichoderma* spp., *P. fluorescens* and *B. subtilis*, respectively, and incubated for 7 days, 48 h and 48h, respectively. After incubation of the antagonists, 5 ml of broth suspension were centrifuged at 5000 rpm for 5 min and the supernatant was stored at 4°C for further procedure. Then, *Ralstonia* spp. suspension of 10<sup>8</sup> cells/ml was prepared as per McFarland 0.5 turbidity method<sup>31</sup> and was swabbed on NA plates. Holes of 5 mm were punched into the agar plate and 40 µl of the supernatant prepared were added separately and the plates were incubated at 27°C for 48 h. Inhibition of *Ralstonia* spp. growth was assessed by measuring the radius in mm of the zone of inhibition (ZOI) after incubation.

For microscopic visualization of the inhibition, CPG agar plates were prepared to provide the most favorable growth to *Ralstonia* spp., and the respective 5 mm mycelial discs of *Trichoderma* species were added in the center of the plate after cotton swabbing from the CPG broth of *Ralstonia* spp. For *B. subtilis* and *P. fluorescens*, the line was streaked parallel to the streak of *Ralstonia* spp. in two different CPG agar plates using dual culture technique. After 72 hr of incubation, live microscopic

examination on the culture plate was done using a digital microscope (Olympus CX-43, Tokyo, Japan). Images were captured to visualize the interaction of the individual strains of biocontrol agents with *Ralstonia* spp.

### Effect of sucrose on the population of biocontrol agents

There is common practice in Nepal of keeping bio-based products in 5–10% (w/v) sucrose solution for 2–4 h before application to the plant. Hence, to evaluate the effect of 5% (w/v) sucrose solution on the cell number (growth) of the biocontrol agents, 1 ml of concentrate containing  $1 \times 10^9$  cells ml<sup>-1</sup> was kept in 5% sucrose solution, made using autoclaved distilled water. Cell count was taken using a Hemocytometer (Reichert, Buffalo, NY, USA) with trypan blue at 1 h, 2 h, 3 h and 24 h to observe the effect on the microbial population.

### Evaluating the effects of biocontrol agents in the field

For the field study, the concentrates containing  $10^9$  cells/ml of the respective biocontrol agent were used. The densities of the cells were determined using Hemocytometer (Reichert, Buffalo, NY, USA)<sup>32</sup>. The TSM broth of all six isolated native *Trichoderma* species viz. AA2, AG3, AKD, A5, A9 and A10 were mixed in equal proportion to prepare 1 liter of concentrate containing  $10^9$  cells/ml. Similarly, two native *P. fluorescens* species viz. PFB and PFS were mixed in an equal proportion to prepare 1 liter of concentrate containing  $10^9$  cells/ml. Concentrate of native *B. subtilis* viz. BS containing  $10^9$  cells/ml was used to analyze the effect of *B. subtilis* as a possible biocontrol agent.

Before the application in the tomato plots at Agro Narayani Farm, the prepared concentrates of biocontrol agents were taken to the field and were further diluted at the rate of 2ml/l of tap water containing 5% (w/v) sucrose. After 2 h of incubation in 5% sucrose water, the diluted solutions were applied in the root of tomato plants at the rate of 100 ml per plant. The processes of applications were repeated every 7 days for 8 weeks (total of 8 applications) by preparing fresh dilutions in 5% sucrose solutions 2 h prior to applications. Effects after the 8 weeks of continuous

application were measured in the field by identifying the number of plants that underwent recovery after treatment. 6 plots were treated with the biocontrol agent and 2 plots were used as controls. Chemical treatment was done in one plot (positive control plot) using the combination of Agricin (9% Streptomycin Sulphate and 1% Tetracycline Hydrochloride) at the rate of 100 ml of 0.1% (w/v) solution per plant from Agricare Nepal Pvt. Ltd., Nepal and Bavistin (50% carbendazim) at the rate of 0.2% (w/v) solution per plant from Crystal Crop Protection Pvt. Ltd., India. For negative control, no treatment methods were selected in one plot.

The treatment plots were designed such that the effects of the individual biocontrol agent and effects of combination treatment can be studied (Table 2).

### Statistical analysis

Statistical analysis of the data was done using IBM SPSS Statistics (ver 23) and figures and data were made through Microsoft Excel 2007 and Microsoft Word 2007.

## Results

### Weather data

Weather data of Chitwan District for temperature, humidity and rainfall were collected from [www.worldweatheronline.com](http://www.worldweatheronline.com) from March 2017 to July 2017 (see Table 3).

The bacterial wilt outbreak was reported in the late May, 2017, when the temperature and humidity level increased. Higher temperature and moisture favors the growth of *Ralstonia* spp.<sup>33</sup>

### Identification of bacterial wilt in the field

From the field examination of the tomato plants, observation revealed that the leaves were flaccid, adventitious roots started to appear on the stem and ooze appeared after dipping the stem in water. Also, field experts from KCC confirmed the presence of bacterial wilt infection, due to their years of experience in plant disease diagnosis.

**Table 2. Design of treatment plot to study the effect of different treatment methods on controlling the bacterial disease.** One plot comprised of 100 tomato plants and 8 plots in total were studied; area of 50 m<sup>2</sup> per plot.

Plot (100 plants/Plot)	Treatment
1	BS ( <i>Bacillus subtilis</i> )
2	TV ( <i>Trichoderma</i> spp. mix)
3	G ( <i>Pseudomonas fluorescens</i> )
4	BS+TV ( <i>Bacillus subtilis</i> and <i>Trichoderma</i> spp. Mix)
5	TV+G ( <i>Trichoderma</i> spp. Mix and <i>Pseudomonas fluorescens</i> )
6	TV+G+BS ( <i>Bacillus subtilis</i> , <i>Trichoderma</i> spp. Mix and <i>Pseudomonas fluorescens</i> )
7	No application
8	Chemical application (Agricin: 9% Streptomycin Sulphate and 1% Tetracycline Hydrochloride + Bavistin: 50% carbendazim)



**Table 3.** Data on average weather over the study period.

Month	Maximum temperature °C	Minimum temperature °C	Average temperature °C	Rainfall (mm)	Cloud (%)	Humidity (%)
March	26	11	21	42.3	16	47
April	32	17	27	184	8	43
May	32	19	28	319.5	13	54
Jun	32	21	28	536	24	71
July	30	21	27	791.8	49	84

Source: [www.worldweatheronline.com](http://www.worldweatheronline.com)

### Isolation and identification of *Ralstonia* spp.

Infected plant saps from six xylems showed similar bacterial colonies on CPG medium. All the colonies were similar to a virulent type, as the appearance was white or cream-colored, irregularly-round, fluidal, and opaque on CPG medium<sup>34,35</sup>. Gram staining and observation using a microscope showed that the bacteria were gram negative, rod-shaped and non-spore forming, which further confirmed that the bacteria was *Ralstonia* spp.

### Evaluating the efficacy of antagonists against *Ralstonia* spp.

Strains tested showed antagonistic effect against *Ralstonia* spp., with inhibition zone radii ranging from 13 to 21.33 mm (Table 4). *P. fluorescens* PFS isolated from *Parthenium hysterophorus* L. rhizoplane soil was most potent compared to other *P. fluorescens* strains. *Trichoderma virens* ATV and *Trichoderma harzianum* ATH provided by TNAU were the least and most potent species. Among six natively isolated *Trichoderma* spp., AA2 isolated from *Parthenium hysterophorus* L. rhizoplane soil was most potent and AKD and A9 were least potent. However, the activities of native *Trichoderma* spp. were satisfactory in the term of inhibition zone shown. *Bacillus subtilis* BS isolated from Bhaktapur top soil did not show satisfactory inhibition activity. The complete photographs showing zone of inhibitions can be retrieved from data availability section.

The effect of biocontrol agents against *Ralstonia* spp. was analyzed at a microscopic level in dark phase using image analyzer (Olympus CX-43, Tokyo, Japan). Figure 1–Figure 3 show the distinct inhibitory effect on the growth and survival of *Ralstonia* spp. caused by different biocontrol agents. From the figures, it can be seen that the most of the pathogenic cells (*Ralstonia* spp.) were either killed or growth was retarded or limited in or towards the region of growth of antagonists as compared to the region away from the growth of antagonists.

The digital images from Figure 1 reveal that the population of *Ralstonia* spp. is significantly less and most of the cells are dead in the region of growth of samples treated with *Trichoderma* spp., compared to the region 4 cm far from the growth of *Trichoderma* spp. as the *Ralstonia* is a rod-shaped bacteria but near the growth region of *Trichoderma*, most of the bacterial

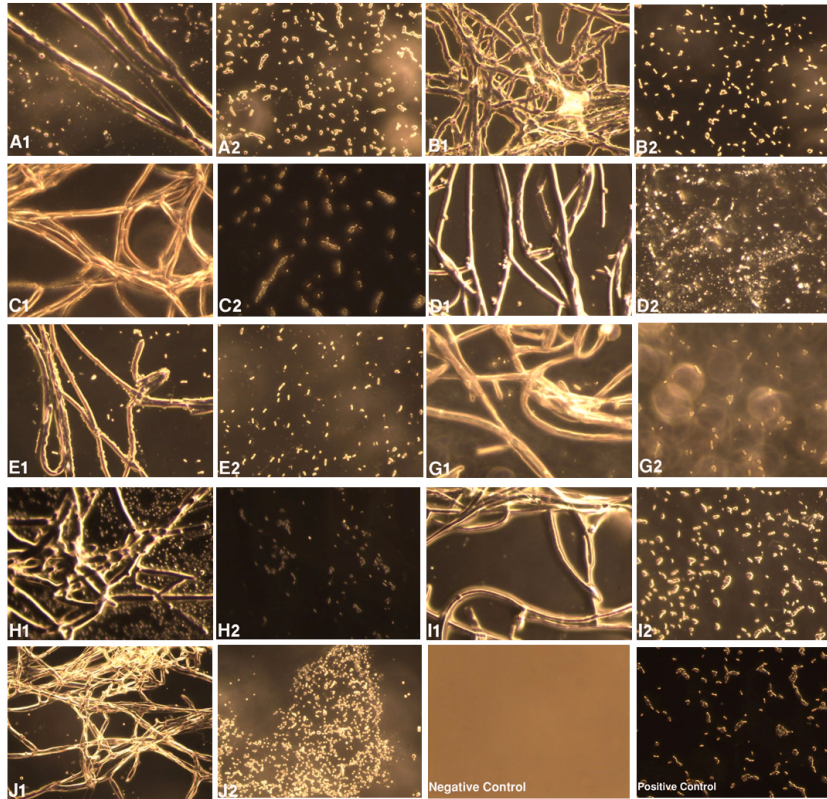
**Table 4.** Inhibition zone made by the isolates used as biocontrol agents against *Ralstonia* spp. using agar well diffusion technique.

All the data are generated using three replications. Values are means ( $\pm$ SE) zone of inhibition (ZOI) in mm against *Ralstonia* spp. (n=3, P <0.05). S30 denotes streptomycin sulphate used at the dilution of 30 mcg. 5 mm diameter of punch hole is included in the data. Code is in reference to Table 1.

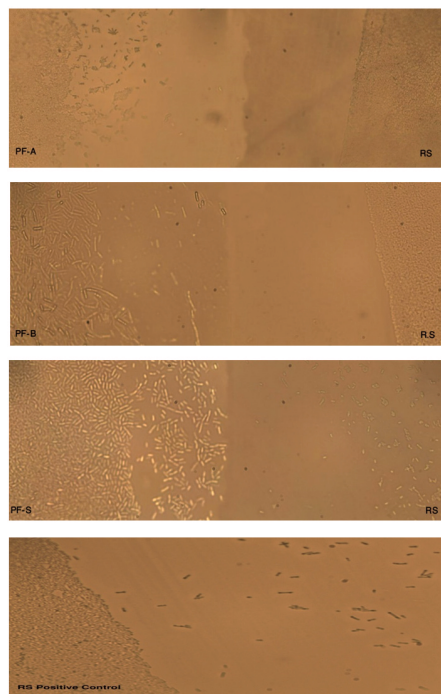
Code	ZOI (in mm)
AA2	20.67 $\pm$ 0.88
AG3	18.33 $\pm$ 0.33
AKD	17.00 $\pm$ 0.58
A5	19.67 $\pm$ 0.67
A9	17.00 $\pm$ 0.58
A10	17.33 $\pm$ 0.66
ATH	21.33 $\pm$ 0.88
ATV	16.33 $\pm$ 0.66
ATA	18.00 $\pm$ 0.58
BS	13.00 $\pm$ 1.53
PFS	22.33 $\pm$ 3.38
PFB	16.33 $\pm$ 0.33
PFA	21.00 $\pm$ 0.58
S30	37.67 $\pm$ 1.33

cells clearly seems to be round and distorted in shape with much lower population density than the region far away This indicates the fact that the *Ralstonia* cells that got inoculated in the plate during the cotton swab were unable to multiply and grow in the zone where *Trichoderma* was growing.

The images from Figure 2 reveal that PF tended to grow on the side of RS, whereas RS tended to restrict the growth towards the PF species. RS positive control (without PF streak nearby)



**Figure 1.** Digital images (400X) of microscopic analysis in dark phase representing the interactions of different *Trichoderma* species against *Ralstonia* spp. A1–J1 shows strains AA2, AG3, AKD, A5, A9, A10, ATA, ATH and ATV, respectively (see Table 1), growing on *Ralstonia* spp., which was cotton swabbed onto CPG agar plates. A2–J2 represents growth of *Ralstonia* spp. 4 cm away from the growth of *Trichoderma* spp., viz., A2, AG3, AKD, A5, A9, A10, ATA, ATH and ATV, respectively, on the plate. Negative control (i.e., blank plate without any swabbing) and positive controls (i.e. *Ralstonia* spp. without *Trichoderma* species) are also included.



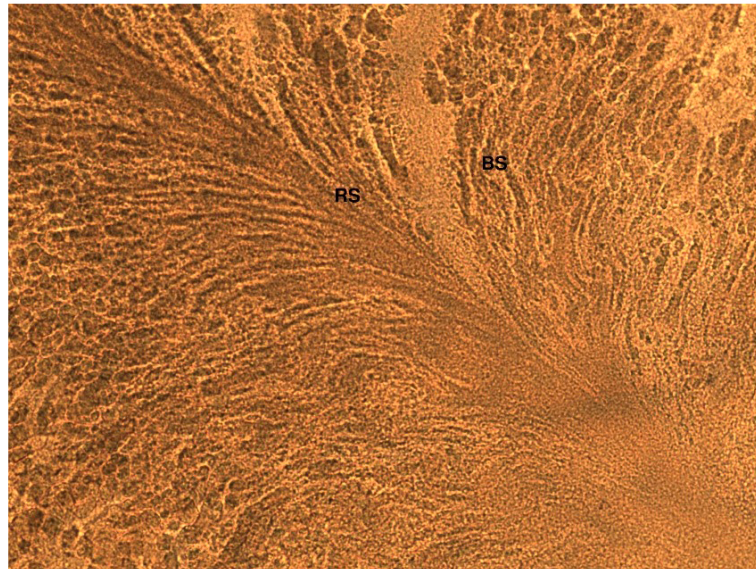
**Figure 2.** Digital images (400X) of microscopic analysis in bright field representing the interactions of *Pseudomonas fluorescens* species against *Ralstonia* spp. PFA, PFB and PFS represent *P. fluorescens* (PF) species (see Table 1), and RS represents *Ralstonia* spp. Both PF and RS were streaked near to each other to see the interaction between the two species.

tended to spread, which confirms the spreading pattern of RS. The microscopic study was to verify a fact that the incompatible species does not grow towards each other and the growth of dominant microbes always surpasses the growth of other recessive microbes. The same phenomenon was observed in the microscopic analysis as *Ralstonia* spp. formed a clear boundary or showed restricted spreading in the region of the streak

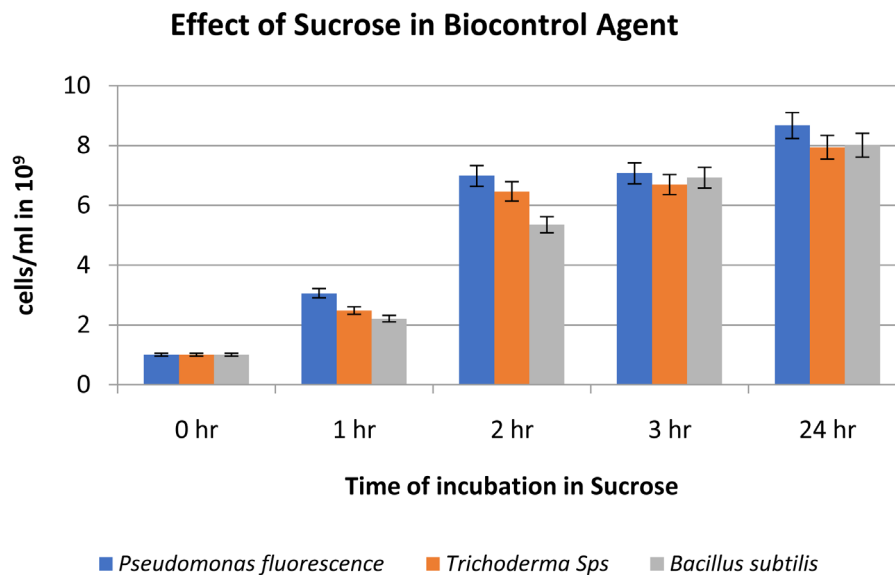
as compared to the Positive control but the streak of *Pseudomonas fluorescens* was easily growing towards the *Ralstonia* spp.

**Effect of sucrose on the population of biocontrol agents**

The effect of sucrose (5% w/v) in the concentrate mixture of individual biocontrol agents was analyzed (Figure 4), which showed that there was a profound increase in the number of



**Figure 3.** Digital image (400X) of microscopic analysis in bright field representing the interaction of *Bacillus subtilis* against *Ralstonia* spp. BS represents *Bacillus subtilis* species (see Table 1), and RS represents *Ralstonia* spp. Both BS and RS were streaked near to each other to see the interaction between two species. BS has completely overgrown the RS streak on the CPG agar plate, which suggests that there has been an interaction between RS and BS and BS is dominant over RS.



**Figure 4.** Effect of sucrose on the growth of *Pseudomonas fluorescens*, *Trichoderma* spp. and *Bacillus subtilis*. An increase in a number of cells of biocontrol agents was seen over time when these agents were kept in 5% (w/v) sucrose solution (n=3). The error bar represents the 5% error in independently performed experiments. The complete data are available in data availability section of this manuscript.



cells of biocontrol agents after 2h of incubation compared with the initial population. The cell count between 2h and 3 h of incubation was not significant as compared with 2 h and 24 h of incubation. Thus, 2 h of incubation in sucrose solution can be considered as optimal time, as lengthier time can result in the growth of contaminant in the solution whilst using tap water in the field.

#### Evaluating the effects of biocontrol agents in the field

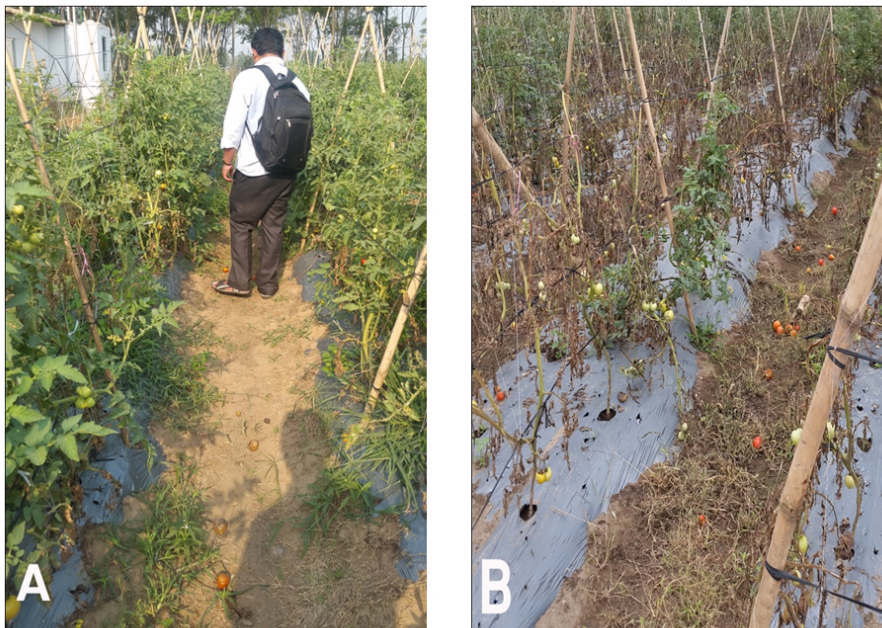
Before applications, 2ml of respective biocontrol agents of ( $10^9$  cells/ml) were incubated in 5% (w/v) of sucrose solution and incubated for 2 hr. The prepared dilution was thus applied at the rate of 100 ml per plant in the root region every week.

8 applications were done over 8 weeks. 8 plots (100 plants/plot) were selected, out of which one plot was used as positive control/chemical treatment plot (Agricin+Bavistin) and one plot as negative control/zero treatment plot (no treatment given).

The results are displayed in [Table 5](#) and show that the application inhibited the bacterial wilt infection (*Ralstonia* spp.) in tomato plants by and the highest rate of plant recovered was 97% from treatment using antagonists, which was comparable with the plant recovery of 94% using the chemical treatment (Agricin and Bavistin). Only 37% of plants were recovered in the plot where no treatment methods were applied. Field Images ([Figure 5](#)) show a clear visualization of growth of

**Table 5. Effect of applying biocontrol agents to tomato plants infected with bacterial wilt.** There was a significant recovery of plants using a mixture of *Trichoderma* species and *Pseudomonas fluorescens*, and the recovery rate was higher than that of chemical treatment. *Bacillus subtilis* did not show significant recovery rate.

S.N.	Treatment plot (100 plants per plot)	No. of plants recovered	No. of plants infected	% recovery
1	BS	84	16	84
2	TV	92	8	92
3	G	96	4	96
4	BS+TV	95	5	95
5	TV+G	97	3	97
6	TV+G+BS	97	3	97
7	Positive control (chemical treatment)	94	6	94
8	Negative Control (w/o treatment)	37	63	37



**Figure 5. Differences in the field between treated and untreated plots.** Field images clearly reveal that the treatment with biocontrol agents has helped to eliminate the bacterial wilt disease in the field after 8 weeks of application. (A) shows the growth and vigor of plants treated with biocontrol agents; (B) shows growth and severity of infection occurred in the untreated plot.

plants and severity of infection in treated and untreated plot. The complete photographs of the field trial can be retrieved from the data availability section of this manuscript.

## Discussion

This research covers the results of the effectiveness of native biocontrol agents in both laboratory and field settings. This research also provides the application strategies of biocontrol agents at the field level. Also, from literature reviews<sup>10–17</sup>, it has been shown that these biocontrol agents can be used to control various other bacterial and fungal diseases, such as Fusarium wilt beside bacterial wilt. Hence, application of these biocontrol agents can also help to prevent other diseases in various crops.

The present results, using microscopy, showed that different species of *Trichoderma* and *Pseudomonas fluorescens* clearly hinders the growth of *Ralstonia* spp., which causes bacterial wilt in tomato plants. *Bacillus subtilis* did not show a significant hindrance. *Trichoderma* spp. secret different compounds against bacteria and also produce various secondary metabolites that promote plant growth and yield<sup>36–38</sup>. *P. fluorescens* produces various compounds that suppress the growth of *Ralstonia* spp. and also induces systemic resistance in the plant<sup>39–41</sup>. *B. subtilis* is well known to induce systemic resistance in plants by secreting various kinds of lipopeptides and secondary metabolites, and this agent also improves plant growth<sup>42–44</sup>. Analysis of data from research, field trials and scientific journal reviews suggests that the application of *B. subtilis* may not immediately show results, but a continuous application of this strain in the agricultural field will slowly induce resistance of plants against pathogenic diseases<sup>42</sup>. Thus, evidence from scientific research show that *Trichoderma* spp. and *P. fluorescens* are effective biocontrol agents against bacterial wilt in compared to *Bacillus subtilis*.

Pre-application of biocontrol agents can successfully prevent the disease attack<sup>45</sup>, induce systemic disease resistance in plants and increase the yield from secondary metabolites secreted by the beneficial bacteria in the biocontrol agent. Thus, we recommend that farmers should continuously apply biocontrol agents in their field so that systemic resistance can get induced in plants and also the plants get protected from invasion of pathogens. Although the cost of production get increased but the farmers can sell their products by considering them as IPM (Integrated Pest Management) product or as an organic product, giving better monetary value for the farmers and health benefits for consumers.

From the results, 2 h incubation of the biocontrol agent in 5% (w/v) sucrose solution was judged as suitable practice being carried out in Nepal for application of biocontrol agents due to two facts. First, it was seen that number of cells of biocontrol agents were increased during the incubation period as observed in **Figure 4**. Second, the water farmers use for drip irrigation generally is unsterilized and comes from an underground source, which may promote the growth of contaminants in sucrose solution if kept for longer periods.

*Trichoderma* spp. and *Pseudomonas fluorescens* provide better results in controlling bacterial wilt in tomato. *Bacillus subtilis* did not perform well in the immediate control of disease. Data from **Table 5** reveals that biocontrol agents can be used as the sole method to control bacterial wilt, and the use of chemical methods can be avoided in the field. Also, combination therapy using both *Trichoderma* spp. and *P. fluorescens* seems to be more effective than treatment using each individual biocontrol agent. A 97% control rate was achieved using combination treatment in the field.

For decades, microbiologists have identified pathogen through the use of phenotypic methods<sup>46</sup>. We authors agree with the fact that 16S rRNA sequencing should be done to identify to the species level for all the microbes used in this manuscript but due to unavailability of the sequencing service in low-income country like Nepal and several unclear quarantine policies in Nepal, researchers in Nepal can only identify to the genus level of microbes by phenotypic methods. If the species has recently diverged then 16S rRNA sequencing alone will not be adequate to assign a species rather it needs additional analytical procedures like sequencing protein coding genes or the intergenic spacer region of the ribosomal gene complex<sup>46</sup>.

## Conclusions

In the present study, *Trichoderma* spp. and *P. fluorescens* seem to be the best biocontrol agents in controlling bacterial wilt induced by *Ralstonia* spp. The zone of inhibition shown by the various antagonists reveals that native isolates were successful in inhibiting the growth of *Ralstonia* spp. The digital microscopy also supports the antagonistic effects of the native isolates.

Also during field application, mixing with 5% (w/v) of sucrose solution and keeping it for 2 h seems to be an effective strategy in better management of bacterial wilt. The application strategies of biocontrol agents with the rate of 100 ml per plant per week successfully recovered the plants from the attack of the pathogen. However, the application rate and amount of biocontrol agents can be varied according to disease severity. Also, the application of the multiple numbers of biocontrol agents can be performed to achieve better results. Hence, native isolates of *Trichoderma* spp. and *Pseudomonas fluorescens* can be used as biocontrol agents to control the bacterial wilt and combined application of these beneficial microbes as bioantagonist can give better results in controlling bacterial wilt infection by *Ralstonia* spp. Results shown by *Bacillus subtilis* were not significant but the scientific researches shows that it can induce systemic resistance in plant with time

## Data availability

**OSF: Raw values of zone of inhibition by antagonist against *Ralstonia* spp.** ZOI is shown in mm and the data were used for statistical analysis. <http://doi.org/10.17605/OSF.IO/9TQCE><sup>47</sup>

**OSF: Raw values of the 5% sucrose treatment.** The average of these values in cells/ml was taken to create the data in the manuscript. <http://doi.org/10.17605/OSF.IO/Q8FVU><sup>48</sup>

Figshare: **Images of the plates showing the zone of inhibition by different antagonists against *Ralstonia* spp.** Clear zone of inhibition obtained by agar well diffusion technique indicates the bioefficacy of the selected bioantagonists against *Ralstonia* spp. <https://doi.org/10.6084/m9.figshare.5562058.v3><sup>49</sup>

Figshare: **Raw digital images (400X) of microscopic analysis representing the interactions of different antagonists against *Ralstonia* spp.** Collection of raw images obtained from digital microscopy in both dark field and bright field microscopy at 400 X zoom. <https://doi.org/10.6084/m9.figshare.5561968.v2><sup>50</sup>

Figshare: **Field Images of plot design showing pictures of before the treatment and effects after the treatment.** There is significant decrease in the occurrence of disease for the treated plots whereas the bacterial wilt has severely affected in the untreated plot. <https://doi.org/10.6084/m9.figshare.5562373.v2><sup>51</sup>

Data are available under the terms of the [Creative Commons Attribution 4.0 license](#) (CC-BY 4.0).

#### Author contributions

SY was actively involved in the generating the data from the field whereas RGC was actively involved in generating data from the laboratory. BRP was actively involved in the generating data from both the field study and the laboratory as well as writing the manuscript. All authors read and approved the final manuscript.

#### Competing interests

No competing interests were disclosed. Author endorsement: Dr. Sushil Thapa confirms that the author has an appropriate

level of expertise to conduct this research, and confirms that the submission is of an acceptable scientific standard. Dr. Sushil Thapa declares he has no competing interests. Affiliation: Texas A&M AgriLife Research, Amarillo, TX 79106, USA.

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*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

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# Open Peer Review

Current Referee Status:  

Version 2

Referee Report 14 March 2018

doi:10.5256/f1000research.15184.r31044



**Teresa A. Coutinho**

Department of Biochemistry, Genetics and Microbiology, Centre for Microbial Ecology and Genomics, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa

"All the colonies were similar to avirulent type, as the appearance was white or cream-colored, irregularly-round, fluidal, and opaque on CPG medium" - if they were avirulent i.e. incapable of causing a disease, then how did bacterial wilt occur in the field?

I suggest you exclude the last paragraph of the discussion concerning access or rather lack of access to a sequencing platform. To overcome this obstacle, researchers in low-income or developing countries should collaborate with those in the developed world (my opinion).

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 14 Mar 2018

**Binayak Raj Pandey**, Agricare Nepal Pvt. Ltd., Nepal

Dear Prof. Dr. Teresa A. Coutinho,

We are very honored that our manuscript was evaluated by you who have published the first report of bacterial wilt caused by *Ralstonia solanacearum* on eucalypts in South Africa and who know the pathogen in detail. Professor, we are really honored !!

You have provided us the insight that how can a research manuscript in a particular topic be polished to a level of scientific readability and acceptance. Your comments in the first version were extremely helpful and i will keep those suggestions in the heart so that i can perform a better scientific research.

Regarding, '**avirulent strain**', it is a typo and it was meant to be "**a virulent**" and hope it will be corrected, thank you for pointing out :-). 'Virulent' and 'Non-Virulent' strains will be more suitable terms to distinguish between them (I think).

I would like to keep the point concerning access or rather a lack of access to a sequencing platform in low-income country like Nepal as this is a fact and somebody may site it too. We are in an era

where Human DNA is being sequenced but in countries like Nepal even the simple microbial sequencing service is not available not even with government and universities. I am not criticizing but this is the situation which we have to accept and act on it for improvements. This is a major reason why researchers have to go to foreign countries to get trained and hands-on-experience for such. Most of the institutions and universities in Nepal are ready to conduct a collaborative research with researchers in developed countries but i think Nepal is not able to seek the attention of researchers around the globe despite being rich in biodiversity. These kinds of initial research may attract researchers to Nepal (I hope so). I would like to share an experience with an Indian Prof. Dr. S. Nakeeran from Tamil Nadu Agricultural University, he quoted that **soil of Nepal is so rich in microbial load that it needs more dilution in order to isolate the desired species.**

It was a challenge for me to publish an acceptable manuscript with limited technologies available in Nepal. Thanks to F1000Research for eliminating the Article Processing Charges for this manuscript, without which we could not have published in this platform.

Thank you again Professor and F1000Research team for your ideas and support.

With Regards,

Binayak Raj Pandey

**Competing Interests:** I do not have any competing interest.

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## Version 1

Referee Report 09 February 2018

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### Dhurva P. Gauchan

Department of Biotechnology, School of Science, Kathmandu University, Kathmandu, Nepal

This manuscript contributes data to the biological control of Bacterial wilt of tomato using microbial consortium which can be turned into commercial product in Nepal. The work is well planned, the methods seem adequate, the results are in a non-negligible amount and discussed on the basis of a convenient literature set. But, the English used in the paper needs to be corrected. If author/s are not expert, they can take help from English expert to rewrite the Sentences. I believe that the manuscript is worthy of being indexed and that it could be accepted after a minor revision, for which I give now some comments, with the aim of helping the authors in obtaining a definitely acceptable version.

#### Title:

Change the title

Evaluation of *Trichoderma spp.* and *Pseudomonas fluorescence* for biological control of Bacterial wilt of tomato.

As Bacillus results are not satisfactory the author supposed to concentrate on only one bacterial and fungal species.

**Abstract:**

Background: *Ralstonia solanacearum* is the major bacterial disease in tomato, which invades the roots of diverse plant hosts and colonizes xylem vessels causing wilt, especially in tropical, subtropical and warm-temperate regions.

Rewrite the sentence: *Ralstonia solanacearum* is the major bacterial disease (*Ralstonia solanacearum* is the not the disease, it is causal organism)

Results: Incorporate the findings of *Bacillus subtilis* (Remove if findings are not up to the mark)

Conclusion: Rewrite the conclusion as it gives confusing information.

**Introduction:**

This study focuses on evaluating the efficacy of different native isolates of *Trichoderma* species, *B. subtilis* and *P. fluorescence* against bacterial wilt disease caused by the pathogen *R. solanacearum* in the tomato plant. The study hypothesizes that the native isolates of *Trichoderma* spp., *B. subtilis* and *P. fluorescence* can be used as bioantagonistic agents to control bacterial wilt (*R. solanacearum*) of tomato. This study tries to reflect the bioantagonistic effects by the microscopic examinations, agar well diffusion technique and by applying the concentrate of biocontrol agents in tomato plots, to calculate their efficiency by comparing them with chemical methods of treatment.

Rewrite and give stress to firm objectives, sentences are poorly written need more accurate writing.

**Methods:**

Write the Latitude: 27.582016 and Longitude: 84.272259 in scientific manner. Trial design is not described (Field Experimentation: Experimental Design). Weather data is not needed. Table: 1 Write full scientific name of plants with authority. Follow strictly ICBN system wherever plant name is used.

Briefly, the stems of the infected plant were washed three times with autoclaved distilled water and then blot dried.

Omit Briefly

Table: 1 Scientific names of source are not uniform. Write complete scientific name of plants with authority. What is the scientific reason to collect samples all from tropical region except *Bacillus* which is collected from mid hill regions of Nepal. Justify.

In this section: Effect of sucrose on the population of biocontrol agents: quote supportive references for this methods.

Choose appropriate statistical tools to evaluate your generated data. Used statistical tools are insufficient.

**Results:**

Only 4 months weather data is not justifiable until it is compared for two consecutive years. Better skip this data which does not show relevance with your work.

*Bacillus subtilis* BS isolated from Bhaktapur top soil did not show satisfactory inhibition activity. When does *Bacillus* give not satisfactory results then better omit *Bacillus* content from the paper.

Table 4. Inhibition zone made by the isolates used as biocontrol agents against *Ralstonia solana cearum*

using agar well diffusion technique.

It is very nice if some supportive pictures are added in this section.

**Discussion:**

The present results, using microscopy, showed that different species of *Trichoderma*, *Pseudomonas fluorescense* and *Bacillus subtilis* clearly hinders the growth of *Ralstonia solanacearum*, which causes bacterial wilt in tomato plants.

AND

Results: *Bacillus subtilis* BS isolated from Bhaktapur top soil did not show satisfactory inhibition activity.

Both statements are contradictory. Justify

Make a common consensus and write obtained results clearly.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Plant and agriculture biotechnology, genetic diversity and plant tissue culture, organic farming

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 17 Feb 2018

**Binayak Raj Pandey**, Agricare Nepal Pvt. Ltd., Nepal

Dear Dr. Dhurba Gauchan Sir,

The comments were extremely helpful to realize few shortcomings of the manuscript. We have briefly detailed and corrected few abnormalities in Abstract, Introduction, Methods, Results and Conclusion sections of the manuscript. Your suggestions has definitely taken the manuscript to next level.



Bacillus subtilis did not show satisfactory results and we have added few points to make it clear that the results were not satisfactory. We have added the clarification of why the Bacillus subtilis was taken from Bhaktapur. The other research articles and our observations (not mentioned in this research) suggest that Bacillus subtilis can induce systemic resistance in a plant with long-term use. Also, the scientific names of the plants were added with authority in version 2.

From next research onwards we will try to incorporate the ideas and suggestions from this manuscript and make also record the weather data for two consecutive years. It's a challenge to work with Biocontrol agents in Nepal as very few genetic tools are available with no option for sequencing and we have to depend on the 3rd world for such services which possess the risk of native potent strains being claimed by others. Hope this lack will end soon in Nepal.

Only few scientist are working in the field of Biocontrol agents and organic farming in Nepal and we are honored that you who fall among those scientist have reviewed our article.

Regards,

Binayak Raj Pandey

**Competing Interests:** We declare that we don't have any competing interest

Referee Report 05 February 2018

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**Teresa A. Coutinho**

Department of Biochemistry, Genetics and Microbiology, Centre for Microbial Ecology and Genomics, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa

The manuscript, particularly the introduction and discussion is poorly written. Each paragraph is an entity on its own and should contain one topic, with a introductory, body and concluding sentence. Many of the paragraphs contain too many topics.

Please correct the spelling of Pseudomonas fluorescens throughout the manuscript.

In the abstract the authors mention that R. solanacearum produces several toxins – toxins are not the major virulence factor in this pathogen – why this sentence was included in the abstract and no where else in the manuscript, is difficult to undertand.

The use phenotypic methods to identify bacteria is no longer sufficient. None of the biocontrol agents or even the causal agent of the wilt symptoms observed where appropriately identified in this study. Based on the latest publications - two Ralstonia species can cause bacterial wilt - R. solanacearum and R. pseudosolanacearum. So which one were they working with? How where the references strains of the biocontrol agents used? Not clear - again their identity needs to be verified by sequencing at the very least (of 16S rRNA).

It is unclear how the inoculum concentrations of the bacterial control agents were determined.

In the experimental plot the authors mention that bacterial wilt was present the previous year. Although I would agree that the pathogen would be present the following year, how did they insure that all areas of the experimental plots were contaminated with the pathogen? Was there 100% loss the previous year?

The use of sucrose in the preparation of the biocontrol agents is concerning. Although the authors noted that it is a common practice in Nepal, the reasons why they would do this need to be provided.

The Figures 1-3 are not very useful. I am not exactly sure what the authors are trying to show. Also, in Figure 1 how do you know the bacterial cells are dead?

There are no error bars on Figure 4.

In Table 4 what do the authors mean by variance.

The discussion and conclusions should be reworked into a comprehensive section. Currently the conclusions are longer than the discussion which is not correct.

**Is the work clearly and accurately presented and does it cite the current literature?**

No

**Is the study design appropriate and is the work technically sound?**

No

**Are sufficient details of methods and analysis provided to allow replication by others?**

No

**If applicable, is the statistical analysis and its interpretation appropriate?**

No

**Are all the source data underlying the results available to ensure full reproducibility?**

No

**Are the conclusions drawn adequately supported by the results?**

No

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.**

Author Response 07 Feb 2018

**Binayak Raj Pandey**, Agricare Nepal Pvt. Ltd., Nepal

Dear Dr. Teresa A. Coutinho,

Thank you for your creative comments. Your comments have helped us to add few points to make

the manuscript more clear. I have tried to address most of the issues in version 2 of the manuscript.

1. Regarding the issue- **The manuscript, particularly the introduction and discussion is poorly written. Each paragraph is an entity on its own and should contain one topic, with an introductory, body and concluding the sentence. Many of the paragraphs contain too many topics.**

-The introduction and discussion section has been re-written. English language has already been reviewed and corrected by native speakers.

2. Regarding the issue- **Please correct the spelling of *Pseudomonas fluorescens* throughout the manuscript.**

-The spelling of *Pseudomonas fluorescens* throughout the manuscript has been corrected. We found some authors using previous spelling as cited in Reference 11 of the manuscript.

3. Regarding the issue- **In the abstract, the authors mention that *R. solanacearum* produces several toxins – toxins are not the major virulence factor in this pathogen – why this sentence was included in the abstract and no where else in the manuscript, is difficult to understand**

-In the abstract, the statement that *R. solanacearum* produces several toxins has been replaced by virulence factors. Thanks for the indication.

4. Regarding the issue- **The use phenotypic methods to identify bacteria is no longer sufficient. None of the biocontrol agents or even the causal agent of the wilt symptoms observed were appropriately identified in this study. Based on the latest publications - two *Ralstonia* species can cause bacterial wilt - *R. solanacearum* and *R. pseudosolanacearum*. So which one were they working with? How where the references strains of the biocontrol agents used? Not clear - again their identity needs to be verified by sequencing at the very least (of 16S rRNA).**

-*R. solanacearum* has been replaced by *Ralstonia* spp. to address the fact that the bacterial wilt was present but which species of *Ralstonia* has caused it needs genetic services to identify. I agree that 16S rRNA is a recently evolved tool and Microbiologist are using Phenotypic methods to identify the microbes from decades. Microbiological tool is still a valid method to identify the microbes to genus level and is still being used as a tool in hospitals, pathology labs and research centers to do so where human life is involved and 16S rRNA is used to identify to the species level. Nepal being a low-income country don't have any sequencing service in any of government or private organization till the date. Hence, the issue with species-level identification has been added in the "Discussion" section of the manuscript and throughout the manuscript, we have mentioned only the genus level of the microbes except *Pseudomonas fluorescens* as they both can be distinguished easily by morphological and microscopic examination and fluorescence that they emit when subjected to UV light. As far as *Bacillus subtilis* is concerned I have mentioned the GeneBank Accession Number for it where I am the first author.

-The wilt symptoms are clearly mentioned and cited properly in the "Isolation and identification of *Ralstonia*" subheading of "Methods section" in the manuscript.

- I have now clearly mentioned that How where the references strains of the biocontrol agents used? in "Isolation of antagonists" sub-heading of "Methods" section.

5. Regarding the issue- **It is unclear how the inoculum concentrations of the bacterial control agents were determined.**

-It has clearly been mentioned in "Evaluating the effects of biocontrol agents in the field" subheading in "Method Section" that a Hemocytometer was used. I don't think we need to mention the protocol of using Hemocytometer to count the cells as it is a very simple procedure adopted even in the diploma curriculums and re-writing such simple protocols will make the manuscript a mesh and will make the readers diverted from the main contents in the manuscript.

6. Regarding the issue- **In the experimental plot the authors mention that bacterial wilt was present the previous year. Although I would agree that the pathogen would be present the following year, how did they insure that all areas of the experimental plots were contaminated with the pathogen? Was there 100% loss the previous year?**

-We did not mention the word "previous year" in any section of the manuscript rather we mentioned "Previous Harvest". In Nepal Harvest can be done 2 times in a year due to pleasant climatic conditions. I have addressed the issue by mentioning that the last harvest was done 3 month before the test started and there was heavy loss in the crop due to wilt infection. We have seen the case that even after 2 years there has been re-occurrence of the wilt in the field, its just 3 months, I think we can buy the fact that the pathogens were present in the field also we tested the soil for presence of pathogen and i have added the same in the manuscript. No, the loss was not 100 % as it has clearly been indicated in Table 5 that with the Negative control (w/o any treatment) there was 63 % loss in the plot. I agree that we should have injected bacterial wilt in the field but it went against our ethics to deliberately destroy the crop of the poor farmer. Hence unlike any other research paper where only field data is taken into account to evaluate the efficiency by injecting the pathogen into the field we thought to evaluate the efficacy in the lab and then comparing the validity of the data from the field. We in the research paper have clearly indicated that the evaluation in the field was done in a natural environment giving all the necessary fertilizers as needed without adultering the pathogen population in the soil where the previous infection occurred. The distance between the treated and untreated plots were merely 0.5 meter in length hence it seems impossible that the pathogens cannot effect the nearby plot. If you seen the pictures from data availability section of the manuscript then you can clearly note that the plot where *Bacillus subtilis* was given as treatment method was effected by the wilt (16 % infection) whereas the infection was 8 % in the plot that was merely 0.5 m from the BS treatment plot. The Negative Control plots (w/o Any Treatment) was also merely 0.5 m from all chemically treated plot but the infection rate was 63 % for Negative Control Plots and 6 % for chemically treated plot. Is that even possible if the pathogen was not present in the soil ?

7. Regarding the issue- **The use of sucrose in the preparation of the biocontrol agents is concerning. Although the authors noted that it is a common practice in Nepal, the reasons why they would do this need to be provided.**

-We have now clearly mentioned and restated in a single paragraph in "Discussion" Section. We just wanted to share our experience that if followed for commercial formulations of Biocontrol Agents gives a better result than just directly spreading it in the field. The other farmers & scientists in another country can adopt the same practice and share their outputs of the trials. The practice



that has been mentioned in the paper merely concern the output that paper is trying to give.

8. Regarding the issue- **The Figures 1-3 are not very useful. I am not exactly sure what the authors are trying to show. Also, in Figure 1 how do you know the bacterial cells are dead?**

- We have now clarified in detail in "Result" section that how the bacterial cells were assumed to be dead and what we are trying to show.

9. Regarding the issue- **There are no error bars in Figure 4.**

-We have changed the style of the graph and also added the error bars. The detail data can be found in the "Data Availability" Section.

10. Regarding the issue- **In Table 4 what do the authors mean by variance.**

- We have removed the variance column, we thought that the variance data could be useful to some statisticians. The detail data can be found in the "Data Availability" Section.

11. Regarding the issue- **The discussion and conclusions should be reworked into a comprehensive section. Currently, the conclusions are longer than the discussion which is not correct.**

-We have reworked both the conclusion and discussion section and made discussion longer than the conclusion.

There are a lot of manuscripts being published on microbes without 16S rRNA sequencing results and we should consider the fact that in some cases 16S rRNA sequencing alone is not sufficient to identify the species. We will try to identify the species in future manuscripts and if the species are novel then we will be happy to publish the results of that too.

Hope I have addressed the major issues in this manuscripts and I assure you that the research from the beginning has been extensively monitored by the professionals from USAID and the manuscript has been reviewed by agricultural scientists from Nepal and USA as mentioned in the manuscript itself at the beginning. I think you can easily understand that it's a challenging work when the biocontrol agents come and the interactions are complex with the pathogens as you have already worked with *Ralstonia* and we have also cited your work in Reference 25 of our manuscript. Hence this manuscript is just a preliminary work to give light on how the interaction can be and such research in Nepal can promote the use of Biocontrol Agents in Nepal and we are continuously thriving to establish the use of these beneficial microbes by working with USAID, Winrock International and FAO in Nepal. The results that we are observing from the fields are astonishing and hope we will soon publish more articles on biocontrol agents where genetic works are done.

With Regards,

Binayak Raj Pandey

**Competing Interests:** There are no any competing interests.

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