Eco-friendly Management of Bacterial Wilt in Tomato Using Dried Powder of the Wild Arid-Land Medicinal Shrub *Rhazya stricta*

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Abstract

Bacterial wilt (BW) disease caused by *Ralstonia solanacearum* species complex is a devastating plant disease that inflicts heavy losses to the large number of economic host plants it infects. In this study, the potential of dried powder of the arid-land medicinal shrub *Rhazya stricta* to control BW of tomato was explored. Both, in vitro and in planta studies were conducted, using different concentrations of dried powder of plant parts, and applied (surface mulched or mixed) to infested soil at 0, 10, and 20 days before transplanting (DBT). Aqueous extract of leaves (16% w/v) was found to be as effective as streptomycin (100 ppm) in inhibiting the in vitro growth of *R. solanacearum*. As evident from the scanning electron micrograph, 16% aqueous extract of leaves produced severe morphological changes, such as rupture of the bacterial cell walls. Results from the greenhouse experiments demonstrated that the higher powder dose (succulent shoot), namely, 30 g/kg of soil mixed with infested soil 20 DBT, was found to be the most effective in controlling BW.

It increased root length (cm), shoot length (cm), and plant fresh biomass (g) by 55, 42, and 40%, respectively, over control plants. Mixing of plant powder with the artificially infested (35 ml of 10⁸ CFU/ml per kilogram of soil) pot soil was better than surface mulching. The 30 g/kg of soil dose mixed with soil increased root length (cm), shoot length (cm), and plant fresh biomass (g) of treated plants by 67, 36, and 46%, respectively, over control plants. A 37% decrease in disease severity over the control was observed with drench application of 30 g of powder per kilogram of soil applied once at 20 DBT. Our results indicated that the dried powder (30 g/kg of soil) of leaves or succulent shoots of *R. stricta*, thoroughly mixed with soil, 20 DBT, could act as an effective control method against BW.

Keywords: *Rhazya stricta*, succulent shoot powder, zone of inhibition, in vivo, in vitro, tomato bacterial wilt, *Ralstonia solanacearum*

Tomato (*Solanum lycopersicum* L.), in terms of production and consumption, is second only to potato in the world. Tomato production is severely affected by bacterial wilt (BW) caused by *Ralstonia solanacearum*. BW, being recently reported from Pakistan, is a relatively new disease there (Begum et al. 2012). The pathogen infects multiple crops of economic importance in the Punjab and Khyber Pakhtunkhwa provinces of Pakistan. It has been reported to cause 21.9% wilt disease incidence on sweet peppers, 16.6% on hot peppers, 13.3% on tomatoes, 10.5% on potatoes and 5.5% on brinjals (Begum et al. 2012; Shahbaz et al. 2015). *R. solanacearum* is considered a species complex. At least six biovars (Hayward et al. 1990) and five races (Fegan and Prior 2005) of this pathogen have been reported. Biovars 1, 2, and 3 have been found in Pakistan, with biovar 3, race 1 being the most aggressive and the predominant one (Begum et al. 2012; Shahbaz et al. 2015; Tahir et al. 2014). Recently, *R. solanacearum* species complex has been reclassified by Safni et al. (2014) into three distinct species: *R. solanacearum* (phylotype II), *R. pseudo-solanacearum* (phylotype I and III), and *R. syzygii* (phylotype IV).

Because of being able to inflict heavy losses to the large number of economically important host plants it infects, BW has been intensively researched, and many possible control measures have been explored in an effort to manage the disease. There have been some very

encouraging results. For example, grafting the scion of popular tomato varieties to interspecific resistant root stock has proven to be a successful practical control measure (Rivard and Louws 2008). Transgenic tomato plants actively expressing EFR and Bs2 genes reduced BW incidence by 50 to 100% in comparison with an untransformed control (Kunwar et al. 2018). Some systemic acquired resistance inducers such as MgO nanoparticles successfully induce systemic resistance in tomato plants against *R. solanacearum* (Imada et al. 2016). However, control of BW is not easy. Some of the main hurdles in the way of effective disease control are the high genetic diversity, soil-borne nature, and wide host range of the pathogen (Nguyen and Ranamukhaarachchi 2010; Wang and Lin 2005), lack of stable BW-resistant varieties (Aslam et al. 2017; Floyd 2007), unavailability of effective commercial biocontrol agents (Whipps and Gerhardson 2007), and lack of commercially available effective chemical controls (Najeeb et al. 2019). The best approach is to use integrated disease management (IDM) to reduce the impact of the pathogen and minimize crop losses. Use of plant-based materials, especially if these plants are abundantly growing weeds, could be an effective and cheap component of IDM against BW (Najeeb et al. 2019). Plant products are environmentally friendly, relatively non-phytotoxic, systemic, and easily biodegradable (Kagale et al. 2004; Mason and Matthew 1996; Tripathi and Dubey 2004).

Plant products in the form of green manures (Cardoso et al. 2006) and dried powders (Din et al. 2016; Naz et al. 2015a, 2015b; Singh et al. 2015), as soil organic amendments prepared from plants with bioactive compounds, and aqueous extracts (Askarne et al. 2012) as seed treatments, or foliar sprays of these plants have already been demonstrated to be effective against different plant diseases. Aqueous extracts obtained from the wild medicinal plant *Adhatoda vasica* were found to be an effective seed treatment against the seed-borne rice bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Govindappa et al. 2011). Aqueous extracts of *Allium sativum* and *Ficus carica* used as foliar sprays were also found to be effective against tomato bacterial speck, bacterial spot, and bacterial canker (Balestra et al. 2009). Singh et al. (2015) used dried powder of *A. vasica* as a mulch and effectively controlled *Meloidogyne incognita* in tomato.

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Medicinal plants have higher amounts of bioactive compounds in comparison with other plants. The products of such plants could be more effective in controlling plant diseases. Additionally, some medicinal plants are wild-growing dry-land plants, available in large amounts and free of cost. Some of such plants include *Withania coagulans* (Najeeb et al. 2019), *Rhazya stricta*, *A. vasica* (Din et al. 2016), *Xanthium strumarium* (Khan et al. 2019b), and *Withania somnifera* (Khan et al. 2019a). Finely ground dried powders of these plants produced encouraging results in *in vitro* tests against BW pathogen.

R. stricta is an important small, evergreen, arid-land medicinal shrub commonly found in Pakistan, India, Afghanistan, Saudi Arabia, Qatar, United Arab Emirates, Iran, and Iraq (Gilani et al. 2007). It is known as “Rangobul” in Urdu and “Vergalu”, Ganderi in Pushto. Various parts of *R. stricta* are used as indigenous medicinal herbal drugs to cure diseases such as syphilis (Ali et al. 2000), toothache, burning feet (Sultana 2006), and so on. The plant has over 100 alkaloids and a few flavonoids (Ali et al. 2000; Sultana and Khalid 2010), and it has an immense antimicrobial potential. Chloroform and methanol extracts of the roots of *R. stricta* had strong antibacterial and antifungal activities against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspergillus terreus*, *Aspergillus flavus*, and *Candida albicans* (Sultana and Khalid 2010). A *R. stricta*-derived compound called tetrahydrosecamine has been shown to have a broad-spectrum antimicrobial activity. Similarly, another chemical called strictanol was also found to be very active (minimum inhibitory concentration of 0.5 mg/ml) against *E. coli* and *P. aeruginosa* (Bashir et al. 1994). The extracts of *R. stricta* have also been reported to have a good nematocidal activity. A concentration of 100 ppm was found to be effective against *Meloidogyne javanica* (Al-Rajhi et al. 1997). In Pakistan, *R. stricta* grows wild, in large quantities, in mountains and in arid wastelands and is available free of cost. This medicinal plant could potentially be used as dried powder organic amendment (OA) or as a green manure OA to eliminate or reduce the soil-borne phase of the BW pathogen. Although the plant has been reported to have strong antibacterial activity (Sultana and Khalid 2010), it has not been tested, to the best of our knowledge, against BW pathogen. Therefore, we proposed that the finely ground powder of this plant should act as an effective soil amendment to control the soil-borne phase of BW. In this research, we tried to achieve two main objectives: (i) to test the effect of different concentrations and times of applications, and (ii) to compare two methods of application of dried powder of *R. stricta* for the management of BW in tomato.

Materials and Methods

Bacterial culture and preparation of inoculum. Pure culture of *R. solanacearum* (biovar 3, race 1) was obtained from the plant bacterial culture collection of the Department of Plant Pathology, The University of Agriculture (UOA), Peshawar, Pakistan. The bacterial pathogen was mass cultured on nutrient agar (NA) for 48 h at 27°C.

Fig. 1. A, Field photograph of *Rhazya stricta* plant; and B, succulent shoot powder of *R. stricta*.

Sterilized distilled water (SDW) was added to each NA plate having bacterial growth, and the bacterial mass was washed off the surface of the plates using a sterilized cotton swab. The culture was resuspended in 0.85% sterile saline (salt) solution (Wai et al. 2013). Using a spectrophotometer (Optima SP 3000+; Japan), the concentration was adjusted to 10^8 CFU/ml ($OD_{600nm} = 0.3$; Lin et al. 2014). This suspension was used for all inoculation experiments.

Plant procurement and preparation of dried powder/aqueous extract. About 80 individual plants of *R. stricta* were collected from the submountainous drylands of district Kohat, Khyber Pakhtunkhwa Province of Pakistan (Fig. 1A). The plants were visually authenticated by a weed botanist (Department of Weed Science, UOA, Peshawar, Pakistan), and specimens were deposited in the UOA herbarium. The plants were washed in running tap water, sorted out as leaves, succulent shoots (soft stems + leaves + flowers/immature pods), and stems, cut into pieces, and shade dried (Mahlo et al. 2010). When brittle-dry, the plant parts were ground to fine powder (2 mm mesh, Kumar et al. 2011). The powders were saved in air-tight plastic containers and stored at 4°C until used (Oguwike et al. 2013) (Fig. 1B). To prepare 8, 12, and 16% w/v aqueous extracts, 8, 12, and 16 g of powders of each plant part were separately mixed with SDW, and the volume was made 100 ml in each case. The suspensions were soaked for 24 h (Frey and Meyers 2010) and then passed through Whatman filter paper (20 μ m), and the filtrates were used for *in vitro* experiments.

In vitro studies. To test the effects of different concentrations of aqueous extracts prepared from different parts of the plant on the *in vitro* growth inhibition of *R. solanacearum*, the agar well diffusion method was used (Perez et al. 1990). Bacterial lawns were prepared by pouring 100 μ l of the bacterial suspension (10^8 CFU/ml) per NA plate and distributing well over the surface (Balestra et al. 2009). Using a sterilized cork borer (9 mm diameter), five wells were cut in each inoculated NA plate. Each well received 100 μ l of either plant extract, SDW (negative control), or 100 ppm of streptomycin (positive control). The plates were incubated overnight at 27°C, and zones of inhibition were measured using a transparent plastic ruler. A completely randomized design (CRD) with six replications was used. The experiment was repeated once. Using *t* test, zones of inhibition of the same treatments in experiments were compared. Finding no significant difference between the treatments of the two experiments, the data were pooled together for analysis.

Scanning electron microscope (SEM) studies. To confirm the effect of the leaf aqueous extracts of the medicinal plant on the growth of the wilt pathogen and to observe any morphological alterations occurring in the bacterial cells as a result of leaf aqueous extract treatment, a SEM (JSM5910, JEOL, Japan) was used. To prepare control and treated cells for morphological observations, small agar pieces were cut out from the relevant inhibition zones and were fixed in 2.5% (v/v) glutaraldehyde (in 0.1 M phosphate buffer; pH 7.2) for 1 h at 4°C. The pieces were then washed three times with phosphate buffer for 10 min and fixed for 2 h with 1% (w/v) osmium tetroxide (OsO_4). This was followed by three washings (in phosphate buffer) for 10 min and dehydration in a graded ethanol series (30, 50, 70, 90, and 95%) for 15 min each. To achieve the critical point, the samples were then subjected to 100% ethanol and liquid CO_2 . The totally dried samples were then coated with gold sputtering and submitted for SEM analysis (Kamonwannasit et al. 2013).

In vivo studies. *Experiment 1: The effect of application time and dose of dried shoots of R. stricta for BW management in tomato.* The purpose of this experiment was to test the effect of the dried powder (succulent shoot) of the medicinal plant *R. stricta* at different concentrations and timing of applications on the control of tomato BW as well as to determine the maximum safe dose of the powder for the tomato crop. The experiment was conducted under screen-house conditions using commercial tomato cultivar Rio Grande. Tomato plantlets were raised in large earthen pots filled with pasteurized (100°C for 6 h; Naz et al. 2015b) soil. When 1 month old, the seedlings were transplanted to 15-cm-diameter perforated plastic pots each having 1 kg of field soil (i.e., sand/clay/silt = 30:23:47) (Aysan et al. 2003; Najeeb et al. 2019). The various powder doses were thoroughly mixed

with soil, and the soil of each pot was moistened before inoculating it with 35 ml of bacterial suspension (10^8 CFU/ml; Lin et al. 2014) poured carefully at the center of each pot (Lin et al. 2014). Non-amended soil served as a control. The experiment consisted of three application times: 20, 10, and 0 days before transplanting (DBT). Each application time involved four dried powder doses: 0, 12, 24, and 36 g per kilogram of potted field soil. There were $3 \times 4 = 12$ treatments, and each treatment was replicated eight times. Each replication consisted of a single plant/pot per treatment. Out of 96 total pots, 32 pots were amended with dried powder at 20 DBT, 32 were amended at 10 DBT, and 32 were amended at 0 DBT. Among each group of 32 pots, eight pots received 0 g of dried powder per kilogram of soil, eight received 12 g, eight received 24 g, and eight received 36 g. The plants were watered and fertilized as per need (Najeeb et al. 2019). CRD with factorial arrangement (3 application timings $\times$ 4 doses of dried powder) was used. The experiment was terminated 60 days after transplanting. The experiment was repeated concurrently at another location (Agricultural Research Institute, Tarnab, Peshawar) in March to April at $28 \pm 5^\circ\text{C}$ (maximum temperature) with no modifications.

Experiment 2: The effect of application method and dose of dried shoots of *R. stricta* for BW management in tomato. In this experiment, we tested whether thorough mixing of the higher dose (30 g/kg of soil) of the dried powder (succulent shoot) with the infested potted soil was any better than its surface mulching (i.e., spreading powder on surface of soil without mixing) in terms of controlling BW. The experiment was conducted under screen-house conditions using commercial tomato cultivar Rio Grande. Nonamended soil served as a control. The experiment consisted of two application methods, namely, thorough mixing of the dried succulent shoot powder with the infested potted soil or its surface mulching. Each application method involved four dried powder doses: 0, 12, 24, and 36 g per kilogram of potted field soil. There were $2 \times 4 = 8$ treatments, and each treatment was replicated eight times. Each replication consisted of a single plant/pot per treatment. Out of 64 total pots, 32 received infested soil thoroughly mixed with powder doses, and 32 received infested soil with a layer of unmixed dried powder on its surface. Among each group of 32 pots, eight pots received 0 g of dried powder per kilogram of soil, eight received 12 g, eight received 24 g, and eight received 36 g. The plants were watered and fertilized as per horticultural recommendations. CRD with factorial arrangement (2 application methods $\times$ 4 doses of dried powder) was used. All other experimental details were the same as above.

Disease severity. Disease severity was recorded four times at 15-day intervals (i.e., 15, 30, 45, and 60 days after inoculation) using a modified four-category scale (Wai et al. 2013). Categories of the scale are 0 = no leaves wilting, 1 = 1 to 25% of leaves wilting, 2 = 26 to 50% of leaves wilting, 3 = 51 to 75% of leaves wilting, and 4 = >75% of leaves wilting (Din et al. 2016). Disease severity index (DSI%) was calculated for each treatment using the following formula (Abdel-Monaim et al. 2011; Bdiya and Dahiru 2006):

$$\text{DSI}\% = \frac{\sum n}{4N} \times 100$$

$\sum n$ is the sum of the ratings of all plants, that is, $\sum (1A + 2B + 3C + 4D \dots)$, where A, B, C, and D... are the number of plants of categories 1, 2, 3, and 4... respectively; N is the total number of plants used for disease rating; and 4 is the highest category of the rating scale. The DSI% data were converted to area under the disease progress curve (AUDPC) values, using the following formula (Madden et al. 2007):

$$\text{AUDPC} = \sum_{i=0}^n \left(\frac{x_{i+1} + x_i}{2} \right) (T_{i+1} - T_i)$$

where n is the total number of assessments, T_i is the time at the i^{th} assessment, and x_i is the infection expressed in quantity at the i^{th} assessment. There were eight plants per treatment, and there was another concurrent experiment (whose data were pooled) having eight plants per treatment.

Monitoring changes in pathogen population in artificially inoculated soil. To find the effect of different treatments on pathogen population per gram of dry soil, initial and final bacterial counts were determined using 10-fold serial dilutions, and the difference was noted. Two soil cores (12×1.1 cm) per pot were taken from tomato rhizosphere, and 16 soil cores of each treatment were mixed together to make a composite sample (Grüter et al. 2006; Schönfeld et al. 2003). Next, three subsamples were then taken from each composite sample. To make 10-fold serial dilution, 5 g of soil was added to 45 ml of SDW, stirred, and thoroughly mixed for a few minutes using a magnetic stirrer. Aliquots of $100 \mu\text{l}$ (10^{-7} dilution) each were poured on tetracycline sodium (TZCNA) selective medium plates (four plates per sample), followed by an overnight incubation at 27°C . To prepare TZCNA, TZC (0.5% w/v) was added (1 ml/100 ml) to autoclaved NA medium (Goszczyńska et al. 2000) before pouring into Petri plates. Bacterial colonies on all four 10^{-7} plates for each subsample were counted, averaged, CFU/g of dry soil calculated, and converted to $\log_{10}$. To record changes in bacterial numbers, % decrease in pathogen population was determined.

Parameters used for data taking. The experiment was terminated 60 days posttransplanting, and data were recorded on (i) disease severity, (ii) shoot length (cm), (iii) root length (cm), and (iv) plant fresh biomass (g). For shoot and root length data, the main shoot and root of each plant were measured using a clear plastic ruler, and for fresh biomass data, the weight of the entire plant was taken using an electronic balance.

Statistical analysis. Data on disease severity, root and shoot lengths, plant fresh biomass, AUDPC, and population dynamics were taken, checked for being normally distributed, and analyzed for regression analysis. All the linear regression analyses and scatter plots were conducted in R software (version 3.4.0) (R Core Team 2019). We developed all the relationships with linear regression models using the *lm* function. Mixed model analysis of covariance (ANCOVA) using the *lm* function was also employed to test treatment effect on slopes by using the explanatory variable and the treatment as fixed effects. Fisher's protected least significant difference (LSD) was also used to test for significant differences between the treatments at $P = 0.05$ (Gomez and Gomez 1984).

Results

In vitro studies. Different concentrations (w/v) of aqueous extracts obtained from finely ground dried powders of leaves, succulent shoots, and stems of *R. stricta* significantly ($P \leq 0.05$) differed from each other in their ability to inhibit the in vitro growth of the bacterial pathogen (Fig. 2). The zones of inhibition produced by the dried powders of leaves, succulent shoots, and stems were 18.1, 16.0, and 13.4 mm, respectively. The higher powder concentration (16% w/v) of all plant parts tested was more effective than the other concentrations and produced a mean zone of inhibition of 18.6 mm. Results

Fig. 2. Inhibition zones of in vitro bacterial growth. Different concentrations (8, 12, and 16% w/v) of dried powders of leaves, succulent shoots, and stems of *Rhizyza stricta* were used to prepare aqueous extracts. One hundred microliters of each extract/sterilized distilled water/streptomycin were poured per well, and the inoculated nutrient agar plates were incubated overnight at 27°C . The experiment was repeated once.

of interactions (plant parts and doses) indicated that 16% (w/v) leaf extract produced the biggest zone of inhibition (21.3 mm). The higher concentration (16% w/v) of leaf powder inhibited the in vitro growth of the bacterium as much as 95% of that inhibited by the standard antibiotic streptomycin (Fig. 3).

Fig. 3. Zones of inhibition (mm). **A**, Effect of different concentrations (w/v) of dried powder of leaves of *Rhazya stricta*: 1 = streptomycin (100 ppm); 2 = 8% conc.; 3 = 12% conc.; 4 = 16% conc.; and 5 = sterilized distilled water (SDW). **B**, Effect of 16% (w/v) concentration of dried powder of different plant parts: 1 = streptomycin (100 ppm); 2 = dried powder of stem; 3 = succulent shoot; 4 = leaves; and 5 = SDW.

Fig. 4. Scanning electron microscope image of *Ralstonia solanacearum* cells: **A**, untreated control cells; and **B**, bacterial cells treated with 16% (w/v) aqueous extracts of *Rhazya stricta* leaves.

SEM observations of the damaged cells. Consistent with the significant in vitro bacterial growth inhibition by the 16% (w/v) aqueous extracts of *R. stricta* leaves, the same concentration of leaves produced enormous morphological alterations in the treated bacterial cells (Fig. 4). In comparison with the untreated cells, the treated cells seem to be severely damaged. They appear misshapen, their cell walls seem degraded, and cell membranes have disintegrated.

In planta studies. *Effect of time of application and doses of dried powder.* Although leaf aqueous extracts were the best in the in vitro test, for the in planta study succulent shoot powder was used, because it is easy and labor-saving to cut, shade dry, and grind succulent shoots in comparison with deleafing the stems and obtaining leaf powder. Moreover, succulent shoots give more powder than leaves. The results of leaf powder and those of succulent shoots were similar in the in vitro studies. Both times of application and different doses of the dried powder (succulent shoot) applied to potted soil differed significantly in terms of enhancing plant growth parameters such as root length, shoot length, and fresh biomass (Table 1). Doses positively predicted the root length, shoot length, and fresh biomass under all the three application times. However, the ANCOVA test of slope difference revealed that time C had a greater slope than B and A in dose relationships with plant growth attributes (Fig. 5; Supplementary Table S1; $P < 0.05$). All growth parameters seemed to be dose-dependent and increased with increasing doses and time of application (days before transplanting or DBT). Best results were obtained when the dried powder was applied at the rate of 30 g/kg of potted soil and 20 DBT, followed by the dried powder applied at the same rate but 10 DBT. As compared with the results of the untreated check (0 g/kg of soil), 55.3% increase in root length, 42.5% increase in shoot length, and 40.6% increase in fresh biomass of tomato plants were recorded when the dried powder was applied at the rate of 30 g/kg of potted soil and 20 DBT. Likewise, all growth parameters were also promoted when the dried powder was applied at the rate of 20 g/kg of soil and 20 DBT. It was noticed that the treatments without the application of the powder (0 g at 0, 10, and 20 DBT) differed from each other regarding shoot length and fresh biomass. Ideally, they should be equal; however, human error such as differences in the fertility of the pot soil, water, and nutrients received by each pot, and so on, might be responsible for that.

Effect of application method and doses of dried powder. The results obtained for plant growth parameters such as root length, shoot length, and plant fresh biomass were significantly different ($P \leq 0.05$) for the different doses of the dried powder used as well

Table 1. Effect of succulent shoot powder of *Rhazya stricta* applied at different intervals of time on growth parameters of tomato plants inoculated with *Ralstonia solanacearum*^a

Application timing (DBT)	Doses (g)				Mean
	0	10	20	30	
Root length (cm)					
20	13.2 ± 1.2 f	21.7 ± 1.7 c	26.5 ± 3.1 b	29.6 a (55.3%)	22.8 A
10	12.8 ± 1.4 f	19.7 ± 2.1 d	22.7 ± 2.1 c	26.2 ± 2.1 b	20.3 B
0	10.7 ± 0.8 g	18.0 ± 1.3 e	20.2 ± 2.4 d	22.7 ± 1.5 c	17.9 C
Mean	12.2 D	19.8 C	23.1 B	26.2 A	
Shoot length (cm)					
20	37.9 ± 4.1 h	45.7 ± 5.3 f	55.7 ± 6.1 c	66.0 a (42.5%)	51.3 A
10	32.0 ± 2.3 i	40.0 ± 4.6 g	51.2 ± 4.4 d	59.0 ± 4.2 b	45.5 B
0	23.5 ± 1.4 k	30.0 ± 4.1 j	38.0 ± 3.2 h	49.0 ± 4.1 e	35.2 C
Mean	31.1 D	38.7 C	48.3 B	58.0 A	
Fresh biomass (g)					
20	33.9 ± 3.3 g	46.2 ± 3.8 d	52.4 ± 4.1 b	57.1 a (40.6%)	47.4 A
10	24.3 ± 3.2 h	35.1 ± 4.2 fg	46.0 ± 2.1 d	50.7 ± 5.7 c	39.0 B
0	14.5 ± 1.3 i	24.7 ± 1.3 h	36.3 ± 2.5 f	42.1 ± 3.1 e	29.4 C
Mean	24.2 D	35.3 C	44.9 B	50.0 A	

^a DBT = days before transplantation. Values in parentheses show % increase over control. The experiment was repeated concurrently at another location in March to April; 28 ± 5°C (maximum temperature) with no modifications. Growth parameters of the two experiments were compared using *t* test. Finding no significant difference, the data were pooled for analysis. Mean values for main effects are bolded and separated by capital lettering. Interactions means are separated by small lettering. Mean values either interactions or main effects showing same lettering are not significantly different ($P \leq 0.05$) as per Fisher's protected least significant difference (LSD) test. Shoot length: application timing LSD = 0.51; doses LSD = 0.59; interaction LSD = 1.03. Shoot length: application timing LSD = 0.73; doses LSD = 0.85; interaction LSD = 1.47. Fresh biomass: application timing LSD = 0.72; doses LSD = 0.83; interaction LSD = 1.44.

as for the two application methods (Table 2). Dose positively related to the root length, shoot length, and fresh biomass under both application methods. ANCOVA revealed method B had higher slope in relationship of dose to root length, shoot length, and fresh biomass (Fig. 6; Supplementary Table S1; $P < 0.05$). Increasing doses of the powder caused significant increases in plant growth parameters. Thorough mixing of plant powder with the potted soil

was found to be superior to surface mulching. Compared with the unamended check (0 g/kg of soil), root length, shoot length, and plant fresh biomass of the plants treated with 30 g/kg of soil (thoroughly mixed with potted soil) were enhanced by 67.3, 36.3, and 46.3%, respectively. Plant growth parameters were the lowest when the dried powder was surface mulched at the rate of 10 g/kg.

Fig. 5. Relationships of plant powder (succulent shoot) doses applied at different times with plant growth parameters root length (A), shoot length (B), and plant fresh biomass of tomato plants (C); area under the disease progress curve (AUDPC) (D); and % decrease in the pathogen population (initial $\log_{10}$ – final $\log_{10}$) (E). Time A = 0 days before transplanting (DBT); time B = 10 DBT; and time C = 20 DBT.

Table 2. Effect of succulent shoot powder of *Rhazya stricta* applied through different application methods on growth parameters of tomato plants inoculated with *Ralstonia solanacearum*^z

Application method	Doses (g)				Mean
	0	10	20	30	
Root length (cm)					
Mixing	10.3 ± 1.3 f	17.8 ± 1.1 d	23.0 ± 2.3 bc	31.5 a (67.3%)	20.6 A
Mulching	11.0 ± 1.8 ef	14.3 ± 1.5 e	20.5 ± 1.6 cd	26.0 ± 2.4 b	17.9 B
Mean	10.6 D	16.0 C	21.7 B	28.7 A	
Shoot length (cm)					
Mixing	41.6 ± 3.1 de	50.5 ± 4.6 c	56.9 ± 6.3 b	65.3 a (36.3%)	53.5 A
Mulching	39.6 ± 2.4 e	43.0 ± 4.4 d	52.1 ± 4.1 c	59.1 ± 4.1 b	48.4 B
Mean	40.6 D	46.7 C	54.5 B	62.2 A	
Fresh biomass (g)					
Mixing	31.4 ± 3.4	40.5 ± 3.2	49.1 ± 3.4	58.5 (46.3%)	44.8 A
Mulching	28.1 ± 2.4	35.5 ± 2.7	42.8 ± 4.1	50.0 ± 5.2	39.1 B
Mean	29.7 D	38.0 C	45.9 B	54.2 A	

^z Values in parentheses show % increase over control. The experiment was repeated concurrently at another location in March to April at 28 ± 5°C (maximum temperature) with no modifications. Growth parameters of the two experiments were compared using *t* test. Finding no significant difference, the data were pooled for analysis. Mean values for main effects are bolded and separated by capital lettering. Interactions means are separated by small lettering. Mean values either interactions or main effects showing same lettering are not significantly different ($P \leq 0.05$) as per Fisher's protected least significant difference (LSD) test. Root length: application method LSD = 1.7; doses LSD = 2.4; interaction LSD = 3.4. Shoot length: application method LSD = 1.1; doses LSD = 1.5; interaction LSD = 2.2. Fresh biomass: application method LSD = 3.7; doses LSD = 5.3; interaction = not significant.

Disease progress over time (AUDPC). *Effect of dried powder doses, application methods, and times on disease severity.* All dried powder doses significantly ($P = 0.05$) differed from the untreated control and from each other regardless of application methods and times (except 20 and 30 g/kg of soil when surface mulched, and 10 and 20 g/kg of soil when applied 20 DBT) (Table 3). Regarding the influence of dried powder doses and application methods (i.e., mulching versus mixing), doses negatively related to AUDPC under both application methods. ANCOVA revealed method B had a smaller slope in dose-AUDPC relationship (Fig. 6; Supplementary Table S1; $P < 0.05$). The higher dose (30 g/kg of soil) thoroughly mixed with the potted soil

produced the best results. It lowered disease severity by 37.12% compared with the untreated control. Likewise, dose-AUDPC prediction produced negative relationships under all three application times (Fig. 5; Supplementary Table S1; $P < 0.05$). The ANCOVA test of slope difference indicated that time C had a smaller slope than B and A in dose relationships with AUDPC. Higher dose and longer application timing decreased percent disease severity more in comparison with lower doses of the dried powder and shorter application timings. The best result was obtained when the dried powder was used at 30 g/kg of soil and applied 20 DBT. The disease severity was reduced by 34.5% compared with control plants.

Fig. 6. Relationships of plant powder (succulent shoot) doses applied through different methods with plant growth parameters root length (A), shoot length (B), and plant fresh biomass of tomato plants (C); area under the disease progress curve (AUDPC) (D); and % decrease in the pathogen population (initial $\log_{10}$ – final $\log_{10}$) (E). Method A = mulching of plant powder; and method B = mixing of plant powder.

Table 3. Effect of dried powder doses (succulent shoot), application methods, and timings on disease severity/AUDPC^z

Experiment	Doses (g)				Mean
	0	10	20	30	
Application timing (DBT)					
20	2,655.0	2,287.5	2,077.5	1,740.0	2,190.0 B
10	2,670.0	2,430.0	2,107.5	1,822.5	2,257.5 AB
0	2,677.5	2,505.0	2,235.0	1,942.5	2,340.0 A
Mean	2,667.5 A	2,407.5 B	2,140.0 C	1,835.0 D	
Application method					
Mixing	2,445.5	2,025.5	1,800.5	1,537.5	1,952.2 B
Mulching	2,482.5	2,167.5	1,972.5	1,815.5	2,109.5 A
Mean	2,464.0 A	2,096.5 B	1,886.5 C	1,676.5 D	

^z DBT = days before transplantation. The experiment was repeated concurrently at another location in March to April at $28 \pm 5^\circ\text{C}$ (maximum temperature) with no modifications. Area under the disease progress curve (AUDPC) values of the two experiments were compared using *t* test. Finding no significant difference, the data were pooled for analysis. Mean values for main effects are bolded and separated by capital lettering. Mean values showing same lettering are not significantly different ($P \leq 0.05$) as per Fisher's protected least significant difference (LSD) test. Application timing: doses LSD = 9.2; timing LSD = 84.2; interaction = not significant. Application method: doses LSD = 121.0; method LSD = 85.62; interaction = not significant.

Influence of application times, methods, and doses of dried powder of R. stricta on population dynamics (CFU/g of dry soil) of R. solanacearum. Doses positively predicted population dynamics under all the three application times. The ANCOVA test showed that time C had a greater slope than B and A in dose relationships with population dynamics (initial log₁₀ – final log₁₀) of *R. solanacearum* (Fig. 5; Supplementary Table S1; $P < 0.05$). The two higher doses, namely, 30 and 20 g/kg of soil (20 DBT), which were statistically at par with each other, significantly ($P \leq 0.05$) reduced the pathogen population (1.57 and 1.34 log₁₀ units, respectively) in artificially infested soil compared with 10 g/kg of soil treatment and the control (Table 4). The same two doses, applied 10 DBT, also achieved significant reduction in pathogen population in comparison with the control. However, the results of all doses applied 0 DBT were non-significant in comparison with control. Similarly, doses positively related to population dynamics under both application methods. ANCOVA revealed method B had a higher slope in dose-population dynamic relationship (Fig. 6; Supplementary Table S1; $P < 0.05$). Regarding the influence of powder application methods in reducing the pathogen population, both mixing and mulching (30 g/kg of soil) produced statistically similar results.

Discussion

Efforts are being made all over the world to replace agricultural pesticides with alternate forms of pest and plant disease control measures. This will safeguard the environment and save humans from the hazards of synthetic chemicals. One way to do so is the exploration of plants, especially abundantly growing wild medicinal plants, as an environmentally friendly source of biopesticides. Several previous researchers have successfully demonstrated that they controlled BW of tomato using plant residues of Chinese gall (Yuan et al. 2012), eucalyptus (Paret et al. 2010, 2012), and thyme (Ji et al. 2005). Here, we evaluated the potential of finely ground dried powder of *R. stricta* against BW of tomato.

There are several reasons to explain the inhibition of bacterial growth by dried powders of *R. stricta*. The most obvious one is the presence of antimicrobial secondary metabolites present in this plant. This plant is especially famous for containing an exclusive array of terpenoid indole alkaloids having diverse biological activities (Ali et al. 2000; Atta-Ur-Rahman et al. 1989; Gilani et al. 2007). Tannins and terpenoids kill bacteria by directly damaging their cell membranes (Mainasara et al. 2012), which results in bacterial growth inhibition (Dorman and Deans 2000). These mechanisms of action are consistent with our SEM studies. The micrograph shows clear morphological changes that occurred as a result of treatment of the bacterial cells with 16% aqueous extract of *R. stricta* leaves. The treated cells, in comparison with untreated cells, seem to be severely damaged. Similar results have been reported by other researchers. When treated with methanol extracts of *Ocimum basilicum* L. and observed in SEM, cells of *Pseudomonas aeruginosa* and *Staphylococcus aureus* appeared

shrunken with their cell walls greatly degraded (Kaya et al. 2008). On treatment of *Staphylococcus epidermidis* cells with leaf aqueous extracts of *Aquilaria crassna*, the cells were found to be swollen, distorted, with ruptured cell walls (Kamonwannasit et al. 2013).

The results of our in planta studies indicated that soil mixing of succulent shoot dried powder of *R. stricta* at different rates significantly augmented plant growth characters, reduced soil bacterial counts per gram, and decreased AUDPC values. Higher dose (20 g/kg of soil) of finely ground dried powder applied 20 DBT was found to be superior to the lower doses applied fewer days before transplanting. A higher amount of the dried powder obviously has more antimicrobial compounds, and longer exposure time helps in more thorough decomposition of the organic amendment, releasing more plant bioactive compounds. Naz et al. (2015a, 2015b) obtained similar results. The researchers used green manures and dried powders of *Fumaria parviflora* as soil organic amendments to control root-knot nematode in tomato. Both forms of the organic amendment were found to significantly enhance plant growth parameters and suppress root-knot nematode (*M. incognita*) populations in a tomato crop. They also found that the higher dose (30 g/kg of potted soil) of dried powder of *F. parviflora* significantly decreased egg masses, females, gall index, and nematode galls and increased yield parameters of the tomato crop under both screen-house and natural field conditions. Aliyu et al. (2011) found that disease severity in cowpeas was significantly reduced and plant growth increased when 12.5 g/kg of neem leaf powder was added to soil for a longer period of time. Additionally, the addition of finely ground powder to soil enhances the activities and abundance of decomposers. Dried powder left decomposing in soil for a longer time would result in increased activities and numbers of soil microbes. This explains the superiority of our 20 DBT results over 10 and 0 DBT. The dried powders usually give better results when applied in the top 0 to 5 cm of soil, but mixing can help in extending these results to deeper layers of soil (Treonis et al. 2010). Furthermore, the addition of dried powder organic amendment to soil also improves soil texture (i.e., physical structure, ion adsorption, soil pH buffering, and water holding capacity) (Braddy and Weil 1999), which helps in disease control and enhanced plant growth characters.

When using plant products, especially those of wild-growing plants, for plant disease control in commercial fields, quality and cost effectiveness may be of concern. *R. stricta* is a wild-growing, evergreen, desert medicinal shrub. Although we do not have any data available regarding chemical differences between *R. stricta* plants growing in different habitats of Pakistan or other countries, several other similar wild-growing plants have been successfully used (Din et al. 2016; Khan et al. 2019a, 2019b; Najeel et al. 2019) for the control of BW. Cost effectiveness, for large-scale field application, might pose a problem for those places where the plant is not available or is scarce, or where labor is expensive. However, in Pakistan, the plant is available on a large scale, free of cost, and in all seasons. It has a long vegetative

Table 4. Effect of application times and methods of dried powder (succulent shoots) of *Rhazya stricta* on % decrease in the pathogen's population (CFU/g of dry soil)^z

Experiment	Doses (g)				Mean
	0	10	20	30	
Application timing (DBT)					
20	0.663 ± 0.12	0.956 ± 0.16	1.340 ± 0.13	1.572 ± 0.12	1.132 A
10	0.672 ± 0.10	0.803 ± 0.11	0.997 ± 0.10	1.220 ± 0.11	0.923 B
0	0.667 ± 0.11	0.764 ± 0.17	0.862 ± 0.10	0.969 ± 0.14	0.815 B
Mean	0.667 C	0.841 C	1.066 B	1.253 A	
Application method					
Mixing	0.735 ± 0.10	1.280 ± 0.19	1.530 ± 0.11	1.920 ± 0.11	1.366 A
Mulching	0.703 ± 0.14	0.940 ± 0.18	1.320 ± 0.19	1.580 ± 0.10	1.135 B
Mean	0.719 D	1.110 C	1.425 B	1.750 A	

^z DBT = days before transplantation. Each value is an average CFU/g of dry soil (initial log₁₀ – final log₁₀). The experiment was repeated concurrently at another location in March to April at 28 ± 5°C (maximum temperature) with no modifications. CFU/g of dry soil values of the two experiments were compared using *t* test. Finding no significant difference, the data were pooled for analysis. Mean values for main effects are bolded and separated by capital lettering. Mean values showing same lettering are not significantly different ($P \leq 0.05$) as per Fisher's protected least significant difference (LSD) test. Application timing: doses LSD = 0.175; timing LSD = 0.151; interaction = not significant. Application method: doses LSD = 0.244; method LSD = 0.173; interaction = not significant.

phase, and its finely ground powder has a long shelf life at room temperature (unpublished data), and the powder is effective in suppressing BW in tomato and possibly other economic crops. These characteristics, coupled with cheap local labor, make the use of this plant a low-cost disease management tool for poor local farmers. Moreover, the dried powder could be target-applied to individual tomato plants, resulting in reduced input costs. The disease control ability of this plant could further be improved by adding simple steps to the disease management strategy for the tomato crop as well other economic hosts of BW. For example, the infested soil into which dried powder is mixed could be plastic mulched for longer times during hot summer days before tomato transplantation. The pathogen is heat sensitive, and soil temperature of 45°C or above for 2 days can kill the bacterium (Kangkiattikajorn et al. 2007). Many areas of Pakistan that commercially grow tomatoes are very hot during the later part of the spring and summer. Therefore, the target temperature of 45°C could be easily achieved through plastic mulching. Powder particle size seems to be important for its bioactivity. Our preliminary results (unpublished data) indicated that the in vitro bacterial growth inhibition zones were significantly increased when very fine dried powder was used. This suggests that more complete mechanical disruption of the plant material results in the releases more antibacterial substances, producing bigger inhibition zones. So, very fine powder could be used to achieve better disease control. To get a disease-free crop, disease-free tomato transplants could be produced in powder-added, moistened, and plastic-mulched seed-bed soils. Rice farmers in Pakistan routinely add neem (*Azadirachta indica*) leaves to seed-bed soils to produce disease-free seedlings (Din et al. 2016).

Toxicity of plant products to the target crop, environment, humans, animals, and beneficial soil microbes is another main concern that should be tackled before their large-scale field application. In our studies, we observed no phytotoxicity of the dried powder of *R. stricta* applied at 30 g/kg of soil to potted tomato plants. In general, plant products are environmentally friendly, relatively nonphyto-toxic, systemic, and easily biodegradable (Kagale et al. 2004; Mason and Matthew 1996; Tripathi and Dubey 2004). Being highly rich in alkaloids (Ali et al. 2000), direct consumption of larger quantities of *R. stricta* might be toxic to humans and animals. However, smaller quantities have been reported to be safe (Adam 1998). The researcher found that oral administration of *R. stricta* leaves at 0.25 g/kg of body weight of sheep did not cause any harmful effects. Moreover, in our studies, the plant product is not directly applied to fruits, vegetable, or other human foods. Rather, it is applied to soil as a finely ground powder, which might quickly leach down and get biodegraded by environmental factors (Naz et al. 2015a, 2015b). To find out whether or not the finely ground powder of *R. stricta* is detrimental to beneficial soil microbes, thorough studies need to be done. Research is underway to answer this important question.

To sum up, our findings suggest that the finely ground powder of *R. stricta* used alone or in combination with other treatments such as dried powders of other medicinal weeds (Din et al. 2016; Najeel et al. 2019) can lead to enhancement of host resistance (Pradhanang et al. 2005) and limited use of chemicals (Santos et al. 2006); it could act as an effective, low-cost disease management tool for BW in tomato and possibly other crops.

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