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MANAGEMENT OF *RALSTONIA SOLANACEARUM* (SMITH) YABUUCHI WILT IN TOMATO (*SOLANUM LYCOPERSICUM* L.) WITH DRIED POWDER OF THE MEDICINAL PLANT *WITHANIA SOMNIFERA* (L) DUNAL.

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Abstract

The potential of dried powders of leaves, stems and succulent shoots of *Withania somnifera* (L) Dunal (applied at different rates and at different application timings), was investigated for the control of bacterial wilt (BW) of tomato. In *In vitro* studies, 15% (w/v) dried powder of leaves produced the maximum (23 mm) zone of inhibition (ZI) followed by the same concentration of dried powder of succulent shoots (i.e., tender shoots plus leaves). The lowest (15 mm) ZI was produced by 5% (w/v) dose of dried powder of stems. The *in-planta* dose of 30g kg^{-1} potted soil (succulent shoot powder) applied 20 days before transplanting (20 DBT) was found to be the best treatment combination. It reduced area under disease progress curve (AUDPC), lowered cfu g^{-1} dry soil, and enhanced plant growth parameters more than the other treatments. The same treatment combination of 30g kg^{-1} potted soil but applied 10 DBT was the second best combination in terms of disease control or yield-contributing plant growth parameters. The poorest plant growth characters were observed in the treatment combination of 15g/kg soil applied 0 DBT. The AUDPC, and cfu g^{-1} dry soil were decreased significantly by the treatment combination of 45g (succulent shoot powder) kg^{-1} soil applied 20 DBT. The plant growth parameters of this treatment combination, however, were lower than those of 30g kg^{-1} soil applied 20 DBT. This suggested that this dose was probably phytotoxic to tomato plants. As compared to the dried powders of other plant parts, leaf-powder (30g kg^{-1} soil) enhanced plant growth characters the most, followed by succulent shoot powder. Although the higher dose of 45 g kg^{-1} soil of leaf powder, like that of succulent shoot powder, declined AUDPC and decreased the cfu g^{-1} dry soil, it failed to enhance plant growth characters as much as those by other treatments suggesting dose-dependent phytotoxic effect. It is concluded from our data that 30g kg^{-1} soil of leaf or succulent shoot powder applied 20 DBT can be an effective component of the integrated disease management (IDM) against BW.

Key words: *Withania*, *Ralstonia*, Bacterial wilt, Medicinal, IDM.

Tomato (*Solanum lycopersicon* L.) is a very important Solanaceous vegetable crop. The Khyber Pathtunkhwa (KPK) province of Pakistan contributes about 0.1618 million metric tons to the total yield of 0.562 million metric tons of tomatoes produced annually in Pakistan. The average yield achieved was 10 and 10.7 tons per hectare in KPK and Pakistan, respectively, which is quite lower than the world average of 36 tons per hectare (MINFAL 2008-2009). Yield losses due to various diseases are one of the major constraints in tomato production. Bacterial wilt (BW), caused by the soil-borne bacterium *Ralstonia solanacearum*, is one of the most devastating bacterial diseases limiting tomato production (Fujiwara *et al.*, 2008). BW is found worldwide, causing more damage in tropical, sub-tropical and warm temperate regions. The bacterium infects plant species belonging to more than 50 plant families including some economically important plants such as potatoes, tomatoes and bananas (Fock *et al.*, 2001). Yield losses in tomatoes, potatoes, tobacco, banana and groundnuts have been reported to be 0-90%, 33-90%, 10-30%, 80-100% and 0-20%, respectively (Elphinstone, 2005). The pathogen is super-variable and is therefore considered as a species complex. Different strains of the bacterium are widely distributed in almost 80 countries of the world and cause economic losses of $ 1 billion per annum (Floyd, 2007). Biovar1, biovar 2 and biovar 3 have been reported to be present in Pakistan with biovar 3 (race 1) being the predominant and the most aggressive. Biovar 2 attacks potato crop primarily in the northern part of the country whereas biovar 3 attacks tomatoes, sweet pepper, hot pepper and aubergine (egg plant). A recent comprehensive survey conducted for recording the prevalence of tomato wilt disease in different areas of the biggest province of Pakistan, the Punjab, indicated that the disease was spreading at alarming rates. Its prevalence was found to be 100% in the districts of Sahiwal, Lahore, Faisalabad, and Sialkot (Begum *et al.*, 2012; Tahir *et al.*, 2014; Shahbaz *et al.*, 2015).

Despite the large number of research trials carried out by researchers all over the world, the efficacy of the management strategies against BW is still very limited. Besides being a broad host range pathogen, the bacterium is able to survive in deeper layers of soil, in weeds, in water and in deeper parts of plant tissues (Wang & Lin, 2005). Because of environmental hazards and public displeasure with chemicals, many researchers evaluated biological control as a possible management option for the control of plant diseases including BW (Whipps, 2001). However, biological control agents (BCAs) perform poorly because of their inconsistent colonization. Additionally, BCAs are often commercially unacceptable as the degree of disease suppression is too low or an uneconomically high rate of inoculum is needed (Whipps & Gerhardson, 2007). Host resistance is, undoubtedly, an effective control option. Bacterial multiplication is suppressed as a result of restricted movement of the pathogen from the proto-xylem or primary xylem to other xylem tissues of resistant tomato

stems (Nakaho *et al.,* 2004). Nevertheless, because of wide host range and high genetic variability of the pathogen, disease resistance in tomato being a quantitative trait, and BW being a high temperature and high humidity tropical disease, it is difficult to produce stable disease-resistant cultivars (Floyd, 2007; Wang *et al.,* 2013; Aslam *et al.,* 2017). This necessitates the search for finding additional control strategies which are cheap, effective, environment-friendly, and relatively stable. Use of plant products as dried powder organic amendments (OAs) (Naz *et al.,* 2015b), green manures (Naz *et al.,* 2015a), soil drenches (Hassan *et al.,* 2009) or foliar sprays of plant extracts (Balestra *et al.,* 2009) is one such option. Plant products, in contrary to synthetic chemicals, reportedly do not pose any threat or pose much lower threat to environment and human health (Harborne, 1998; Verma & Dubey, 1999; Gottlieb *et al.,* 2002). Many control options, including plant-based ones, could be integrated together to effectively control a devastating disease like BW. Integrated disease management (IDM) has been demonstrated to be successful in reducing BW from 20 to 100% (Anith *et al.,* 2004). Using thymol, palmarosa, and lemon-grass oil as bio-fumigants, the incidence of BW of tomato was significantly reduced and higher yields were achieved (Pradhanang *et al.,* 2003; Ji *et al*., 2005, Paret *et al.,* 2010).

The use of OAs not only suppresses BW but also improve chemical, physical, and biological properties of soil which positively affect growth of plants resulting in higher yields. Degradation of organic matter in soil releases natural anti-microbial chemical substances which are inhibitory to the viability and survival of pathogens. Moreover, decomposing organic matter releases carbon resulting in increased activities of competing soil microbes (Bailey & Lazarovits, 2003; Cardoso *et al.,* 2006). The incidence of BW was reduced up to 53% by using organic mixture and Actigard. It was suggested that either the induction of systemic resistance or the antibacterial properties of the OA or both, suppressed BW (Anith *et al.,* 2004).The dry amendment of soil by the addition of dried plant powders as well as the application of green manures were found to be equally effective in controlling plant diseases (Naz *et al.,* 2015a, b). Use of medicinal plants to control plant diseases has an extra advantage of possessing larger amounts of anti-microbial natural compounds which result in more effective suppression of plant pathogens (Din *et al.,* 2016). *Withania somnifera* is an ever-green herbaceous medicinal weed growing in barren and waste lands in Pakistan. The plant is available in large quantities year round and is free of cost. It possesses magnificent anti-bacterial properties. It has been used for the treatment of a number of human diseases (Verma & Kumar, 2011). It is a commonly used medicinal plant in subcontinent (Khan *et al.,* 2010). However, the potential of *W. somnifera* for the control of BW has not been previously explored. Therefore, we tested the possibility of using dried powder of *W. somnifera* as an effective component of IDM against BW. Since different types and amounts of bioactive compounds are present in different parts of plants (Kolapo *et al*., 2009; Naz *et al.,* 2016), we also tested the influence of dried powders of stems, leaves, or succulent shoots of *W. somnifera*, applied at three different times, for the control of BW.

Materials and Methods

Preparation of water extracts of plant's parts: Plant of the medicinal weed, were collected from waste lands around the University Research Farm, and authenticated by a weed botanist. To prepare finely ground powders of stems, leaves and succulent shoots i.e., tender shoots with leaves, the plant parts were separated, washed with tap water and shade-dried (Mahlo *et al.,* 2010). When brittle dry, the plant parts were finely powdered. Different concentrations (5%, 10%, and 15% w/v) of the parts of the plant were prepared by separately soaking the required amounts of their finely ground powders in sterilized distilled water for 48 hours (Frey & Meyer, 2010). The soaked samples were then filtered through three layers of cheese cloth and the filtrate was used for *In vitro* bacterial growth inhibition.

Bacterial culture and preparation of inoculum: Pure culture of the bacterial pathogen was obtained from the bacterial culture bank of the Department of Plant Pathology, The University of Agriculture, Peshawar, Pakistan. The culture was grown on CPG (Casamino acid = 1g; Peptone = 10g; Glucose = 5g; Agar = 17g per 1 L medium) having 5 ml of 1% TTC (2, 3, 5-triphenyl tetrazolium chloride) (Kelman 1954) for 48h at 30ºC. The 1% stock solution of TTC (autoclaved for 5 min at 121ºC and stored at 4ºC or frozen) was added when the autoclaved medium cooled down to 55ºC. A well isolated EPS^+ (having extracellular polysaccharide) colony, white or pink in color, was picked and mass-cultured on NA (nutrient agar: 0.5% peptone, 0.3% beef extract/yeast extract, 1.5% agar, 0.5% sodium chloride and 1 L distilled water) at 30ºC for about 24 h (Fig. 3). Using sterilized distilled water (SDW), the surfaces of the NA plates having bacterial growth, were flooded, the growth was scrapped off using sterilized cotton swabs (Wai *et al.,* 2013) and the bacterial suspension was adjusted to 10^8 cfu/ml i.e., OD_{600} = 0.3 (Lin *et al.,* 2014). This bacterial suspension was then used for all subsequent experiments.

***In vitro* bacterial growth-inhibition:** The ability of the plant's decoction (different concentrations and different parts of the plant) to inhibit the bacterial growth on NA medium, agar well diffusion technique was used (Perez *et al.,* 1990). To obtain a homogeneous bacterial lawn, 100µl of the suspension (OD_{600} = 0.3 corresponding to 10^8 cfu/ml) were poured on each NA plate (Balestra *et al.,* 2009) and spread uniformly using sterile cotton swabs. Next, three wells were punched in the medium using sterile cork borer (9 mm diameter) and 100 µl of each concentration were poured into different wells. Positive control consisted of streptomycin (100 ppm) and for negative control, 100 ul SDW was used. All the plates were incubated for 24 h at 28ºC. The resulting zones of inhibition (ZI) were measured using clear plastic ruler. CRD (completely randomized design) was used with six replications. The experiment was repeated two times.

Studies under screen house conditions

Procuring tomato transplants: Tomato nursery (cultivar Rio-grande) was raised in earthen pots by direct sowing

of seeds. The plants were watered and fertilized as per horticultural recommendations. Plastic pots (15 cm diameter) were filled with 1 kg field soil each (Aysan *et al.,* 2003) and were used for transplanting (1 plant/pot).

Experiment 1

Impact of application timings of dried powder and doses: To test the hypothesis whether there was any impact of time of application or the dose of the dried powder (succulent shoot), three different timings and four different doses were tested. The timings included 0 days before transplanting or DBT, 10 and 20 DBT and the doses were 0g, 15g, 30g, and 45g/kg potted soil. The powder doses were thoroughly mixed with the soil. The experiment consisted of 12 treatments (3 timings x4 doses) and each treatment was replicated six times. CRD with factorial arrangement was used for the experiment. Twenty four pots were amended with the dried powder 0 DBT, 24 pots 10 DBT and 24 pots 20 DBT. Within each application timing, there were four sets of 6 pots each. The first set received 0g, second 15g, third 30g and fourth 45g dried powder/kg soil. The experiment was ended 60 days after transplanting. The data were taken on (i) disease severity, (ii) plant height (cm), (iii) root length (cm), (iv) plant fresh bio-mass (g) and (v) plant dry bio-mass (g). The experiment was repeated once.

Experiment 2

Impact of dried powder of plant parts and doses: In this experiment, it was tested whether the dried powders of leaves, stems and succulent shoots of the plant differed from each other in terms of their ability to control BW. It was also tested if there was any difference between different doses (0g, 15g, 30g, and 45g/kg soil) of the dried powders of these parts. The dried powders were mixed with potted field soil (1 kg/pot) 10 DBT. There were 4×3=12 treatments and each treatment was replicated six times (12x6 = 72) using CRD with factorial arrangement. Twenty four pots each were amended with stem powder, leaf powder and green top powder. Within each group of 24 pots, 6 pots each received 0g, 15g, 30g and 45g powder/kg soil. The experiment was terminated 60 days after transplanting and data were taken on various parameters as described before. The experiment was repeated once.

Infestation of soil and disease severity rating: To artificially infest the soil, each pot containing 1 kg pre-moist field soil (Aysan *et al*., 2003) was poured with 35 ml of the bacterial suspension at the center of the pot. The different doses of the powders were mixed with the field soil before its infestation. Crop husbandry, after transplanting, was done as per horticultural recommendations. Data (on disease severity) were taken at 20 days interval. For disease severity, the 1-5 rating scale of Wai *et al.,* (2013) was used and the disease index (DI%) for each replicate was calculated according to Abdel-Monaaim *et al.,* (2011). Area under Disease Progress Curve (AUDPC) were calculated as per Madden *et al.,* (2007).

Population dynamics of *R. solanacearum* in soil: To test if different doses of the dried powders of stems, leaves and succulent shoots of the medicinal plant applied at different times before transplanting had any impact on the population of the bacterial pathogen in artificially infested soil, three soil cores (per pot) were taken from the vicinity of the roots at the depth of 12 cm using10 mm diameter cork borer. All soil cores (eighteen) of each treatment were mixed together to make a composite sample (Schonfeld *et al.,* 2003; Gruter *et al.,* 2006). Three sub-samples were taken from each composite sample, and ten-fold serially diluted up to 10^{-7}. Aliquots of 100 µl each from 10^{-7} of each treatment were poured/plate of TZCNA selective medium (Goszczynska *et al.,* 2000). The plates were incubated for about 48 hours at 28°C. Off-white colonies with a red centre were counted and cfu/g of soil were calculated.

Statistics

Data on disease severity, root and shoot lengths, and plant's fresh and dry biomass were analyzed using StatistiX 8.1(Campbell & Madden, 1990). Treatment means were separated using Fisher's Protected Least Significant Difference (LSD) test at p = 0.05 (Gomez & Gomez, 1984).

Results

***In vitro* antibacterial assay:** The aqueous extracts of leaves and succulent shoot were found to be statistically at par with each other and with streptomycin (100 ppm) in terms of inhabiting the *In vitro* bacterial growth Fig. 1). Although the *In vitro* bacterial inhibition was found to be generally dose-dependent, the 10% and 15% concentrations of the aqueous extracts of leaves, succulent shoot and stems produced statistically similar results. The 15% (w/v) aqueous extract of leaves and succulent shoots produced inhibition zones of 23.0mm and 22.5mm, respectively. The 5% (w/v) aqueous extract of stem powder produced the lowest (15mm) inhibition zone (Fig. 2). The repetition experiments produced similar results.

***In-planta* antibacterial tests**

Effect of time of application and doses: To test the hypothesis that the *In vitro* anti-bacterial activity of *W. somnifera* could be translated to *in planta*, 4 dried powder (succulent shoot) doses viz. 0g, 15g, 30g, 45g kg^{-1} soil were mixed with potted field soil 20 days before transplanting (DBT), 10 DBT and 0 DBT. In general, it was found that the application time of 20 DBT was better than the other application times. The treatment combination of 30g kg^{-1} soil applied 20 DBT enhanced tomato shoot length, root length, fresh biomass and dry biomass by 38.87%, 53.11%, 40.54% and 42.14%, respectively. Plant growth parameters increased with increasing doses and times of application (except for the treatment of 45g kg^{-1} soil) (Table 1). The powder dose of 30g kg^{-1} soil was, however, statistically not different from the smaller dose of 15g kg^{-1} soil in terms of all plant growth parameters except root length.

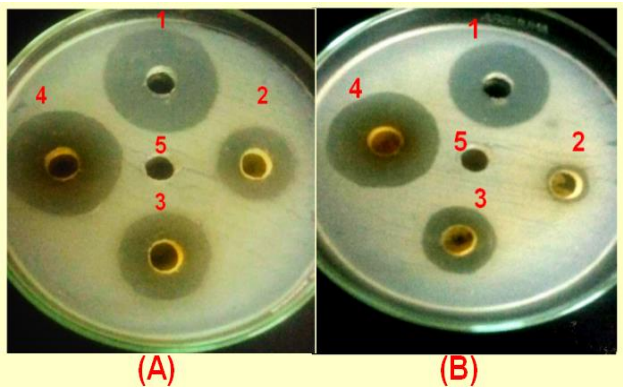

Fig. 1. Zones of inhibition (mm) produced by three different concentrations (2= 5%; 3= 10%; 4= 15%) of leaves (A), and 15% concentration of different plant parts (2= stems; 3= succulent shoot; 4= leaves) of *Withania somnifera*. 1 = Positive control i.e., streptomycin (100 ppm) ; 5= Negative control (water). Bacterial lawns were prepared by streaking NA plates with sterilized cotton swabs dipped in bacterial suspension of 10^8 cfu/ml (OD_{600} = 0.3). Plates were incubated overnight at 28°C.

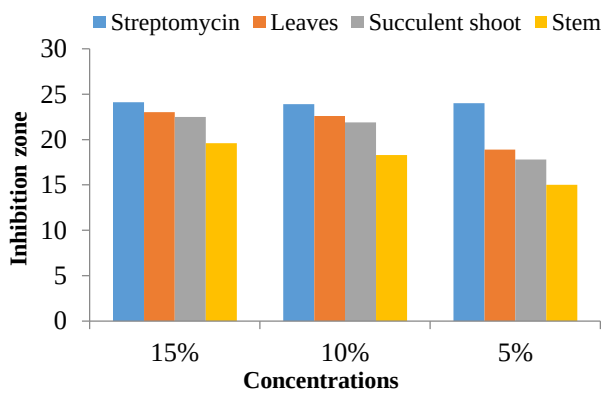

Fig. 2. *In vitro* growth inhibition of *R. solanacearum* by aqueous extracts prepared from dried powders of leaves, stems and succulent shoots of *W. somnifera,* 24 h after incubation at 28°C.

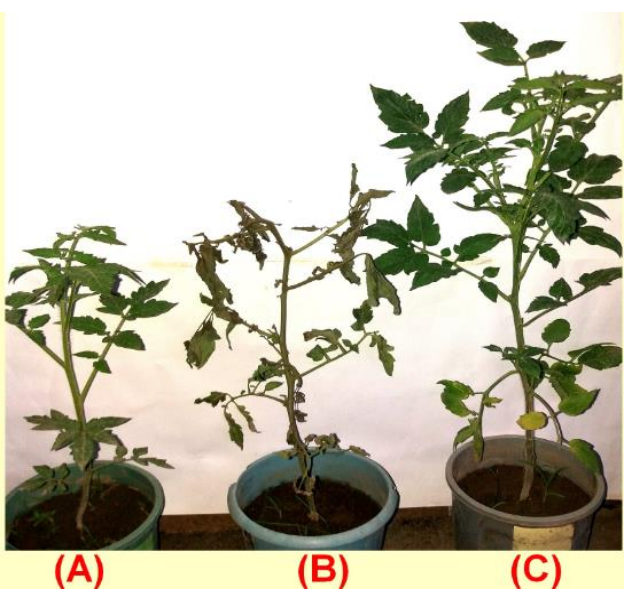

Fig. 3. Response of tomato plants (Rio Grande) inoculated with *R. solanacearum*, to different levels of dried powder of succulent shoot of *Withania somnifera*. A = Tomato plant showing stunted growth due to possible dose-dependent phytotoxicity of 45 g kg^{-1} soil dose. B = Wilted tomato plant (untreated control). C = Healthy tomato plant treated with 30 g kg^{-1} soil.

Effect of plant parts and doses: As compared to the un-treated control plants (0g kg^{-1} soil), the 15g kg^{-1} and 30g kg^{-1} soil powder doses of stem, leaves and green top significantly improved the plant growth parameters. The higher dose of 45g kg^{-1} soil caused stunting of plants. The improvements in the shoot length, fresh and dry biomass due to treatment combination of 30g kg^{-1} soil leaves powder was at par with those of 30g kg^{-1} soil green top powder (Table 2). The treatment combination of 30g of leaves powder augmented height of plant (cm), length of root (cm), fresh and dry biomass (g) of plant by 36%, 59.45%, 40.57% and 41.86%, respectively.

Effect of dried powder in reducing cfu g^{-1} of potted soil: In comparison with the untreated control treatments, the influence of the different doses (15, 30 and 45g kg^{-1}soil) of the dried green-top powder applied at three different timings in reducing the number of cfu g^{-1} of the artificially infested soil was significant. However, the results of the two higher powder doses (30 and 45g kg^{-1}soil), regarding the decrease in the pathogen population, were statistically at par with each other, regardless of the time of application. The lower dose (15g kg^{-1} soil) also produced statistically similar results as those produced by 30g kg^{-1} soil dose ($p \leq$ 0.05). The application time of 20 DBT was found to be better than 0 DBT for both 45g kg^{-1} soil and 30 g kg^{-1} soil (Table 3).

The dried powders (stems, leaves and succulent shoot) of *W. somnifera* applied at 15, 30 and 45g kg^{-1}soil, also decreased cfu/g of soil as compared to untreated control treatments (Table 3). The higher dose (45 g kg^{-1}soil) of leaf and stem powder significantly reduced bacterial counts per g soil. All other doses of leaves, stem and green top powder gave results which were statistically not different from each other, though they were different from control treatment.

AUDPC (area under disease progress curve)

Effect of application times, plant parts and doses of dried powder on disease severity: All the three doses (15, 30, and 45 g kg^{-1} soil) of dried powder of all plant parts differed significantly from the control treatments regardless of application times (Table 4). Percent disease severity was decreased when higher doses and longer application times were used. The higher dose (45 g kg^{-1} soil) of leaves powder proved to be better than the same dose of the dried powders of other plant parts in bringing down the AUDPC values. It reduced AUDPC value by 38.27% as compared to that of the control treatment. Among the three tested application times, 45 g kg^{-1} soil of green top powder applied 20 DBT produced the best results. It reduced AUDPC values by 37.34%.

Table 1. Effect of dried powder of succulent shoots of *W. somnifera* applied at different intervals of time on growth parameters of tomato plants (inoculated with *R. solanacearum*) 60 days after transplanting.

Application timing	Doses (g)	Shoot length (cm)	Root length (cm)	Fresh bio-mass (g)	Dry bio-mass (g)
20 DBT	0	40.60 EFG	15.18 FG	35.05 BCD	5.60DEF
	15	**57.225 AB (29.05%)**	**26.62 BC (42.97%)**	**46.48 AB (24.59%)**	**8.40 AB (33.33%)**
	30	**66.425 A (38.87%)**	**32.38 A (53.11%)**	**58.95 A (40.54%)**	**9.68 A (42.14%)**
	45	45.80 CDE	15.11 FG	25 DE	4.42 EF
10 DBT	0	33.20 GH	14.52 G	24.88 DE	4.95DEF
	15	41.80 DEFG	22.40 DE	36.75 BCD	6.60BCD
	30	**50.35 BCD (34.06%)**	**29.34 AB (50.51%)**	**48.50 AB (48.70%)**	**7.88ABC (37.18%)**
	45	39.08 EFG	19.81 E	27.32 CDE	4.38 EF
0 DBT	0	25.80 H	13.21 G	15.15 E	3.90 F
	15	34.40 FGH	19.05 EF	26.90 CDE	4.80DEF
	30	43 CDEF	25.02 CD	38.65 BC	6.08CDE
	45	41.60 BC	20.88 E	28.95 CD	5.18DEF
LSD values		**9.69**	**3.99**	**13.59**	**2.0019**

Means (n = 6) with the same English alphabet letter (column-wise) are not significantly ($p \leq 0.05$) different from one another based on Fisher's protected LSD test. Values in parenthesis indicate % increase over control. The experiment was repeated once with similar results

Table 2. Effect of different doses of dried powders of stems, leaves and succulent shoots of *W. somnifera* applied 15 DBT on growth parameters of the inoculated tomato plants 60 days after transplanting.

Plant parts	Doses (g)	Shoot length (cm)	Root length (cm)	Fresh bio-mass (g)	Dry bio-mass (g)
Stem	0	31.075 E	11.73 G	17.75 I	2.65 D
	15	39.38 DE	20.02 DE	25.85 H	5.22 CD
	30	48.12 BCD	28.75 BC	33.95 EFG	7.82 BC
	45	53.32 BC	17.65 EF	42.05 BCD	6.08 C
Leaves	0	40.08 DE	15.11 EFG	32 FGH	7.22 BC
	15	**52.40 BC (23.51%)**	**26.97 C (43.97%)**	**45.50 BC (29.67%)**	**9.82 AB (26.47%)**
	30	**62.625 A (36%)**	**37.27 A (59.45%)**	**53.85 A (40.57%)**	**12.42 A (41.86%)**
	45	46.02 CD	14.53 FG	27.58 GH	5.05 CD
Green top	0	34.98 E	13.34 FG	32.60 FGH	2.65D
	15	46 CD	23.88 CD	40.95 CDE	7.52 BC
	30	**55.15 AB (36.57%)**	**32.72 B (59.22%)**	**48.800 AB (33.19%)**	**10.12AB (73.81%)**
	45	50.50 BC	16.05 EFG	35.68 DEF	5 CD
LSD values		**9.0827**	**5.1660**	**7.4811**	**3.1909**

Means (n = 6) with the same English alphabet letter (column-wise) are not significantly ($p \leq 0.05$) different from one another based on Fisher's protected LSD test. Values in parenthesis indicate % increase over control. The experiment was repeated once with similar results

Table 3. Effect of succulent shoot powder (applied at 0 DBT, 10 DBT and 20 DBT); and leaf, stem and succulent shoot powders (applied at 15 DBT) doses of *W. somnifera* on the population dynamics (cfu/g of soil) of *R. solanacearum* 40 days after soil inoculation.

Doses		Application timing (succulent shoot)			Plant parts		
		cfu/g of soil			cfu/g of soil		
		0 DBT	10 DBT	20 DBT	Leaves	Stem	G. top
0 g	0th day	10.238 A	10.224 A	10.224 A	10.271 A	10.251AB	10.285 A
	40th day	9.87 A	9.87 A	9.87 A	9.91 A	9.89 AB	9.97 A
15 g	0th day	**10.200 AB**	10.154 BC	10.108 CD	10.181 DEF	10.232 BC	10.202 CD
	40th day	**9.76 B**	9.75 B	9.68 BC	9.67 DE	9.81 BC	9.73 CD
30 g	0th day	10.114 CD	10.068 DE	**10.023 EF**	**10.153 FGH**	10.197 CDE	10.167 EFG
	40th day	9.76 B	9.70 BC	**9.63 CD**	**9.45 G**	9.60 EF	9.51 FG
45 g	0th day	10.058 DE	**10.013 EF**	**9.968 F**	**10.120 H**	10.161 FG	**10.136 GH**
	40th day	9.69 BC	**9.63 CD**	**9.55 D**	**8.56 I**	8.71 H	0.0953 HI

Means (n = 6) with the same English alphabet letter (column-wise) are not significantly ($p \leq 0.05$) different from one another based on Fisher's protected LSD test. Values indicate cfu/g dry soil converted to log. Pot soil was inoculated with bacterial suspension at the time of incorporation of the dried powder. The experiment was repeated once with similar results

Table 4. Effect of succulent shoot powder (applied at 0 DBT, 10 DBT and 20 DBT); and leaf, stem and succulent shoot powders (applied at 15 DBT) doses of *W. somnifera* on AUDPC values.

Doses	Application timing (succulent shoot)			Plant parts		
	AUDPC			AUDPC		
	0 DBT	10 DBT	20 DBT	Leaves	Stem	Green top
0g	1766 A	1670 A	1655 A	2143 AB	2252 A	2216 A
15g	1491 B (18.43%)	1470 BC	1391 BCD	1851 DEF	2013 BCD (11.84%)	2033 BC
30g	1378 BCD	1345 CD	1308 DE	1741 F	1903 CDEF	1918 CDE
45g	1265 DE	1263 DE (32.19%)	1205 E (37.34 %)	1550 G (38.27%)	1793 EF	1766 EF (25.47%)
LSD values		**136.84**			**165.85**	

Means (n = 6) with the same English alphabet letter (column-wise) are not significantly different ($p \leq 0.05$) from one another based on Fisher's protected LSD test. Values in parenthesis indicate % decrease over control. AUDPC = Area under disease progress curve. The experiment was repeated once with similar results

Discussion

Bacterial wilt (BW), is a soil-borne, water-borne and seed-borne disease (Huet, 2014). Lack of efficient chemical control (Saddler, 2005; Denny, 2006), ability of the pathogen to shelter itself in the deeper layers of soil, in xylem vessels of host plants and weeds (Wenneker *et al.,* 1999) and environment-linked nature of resistance (Hayward, 1991), makes it very difficult to control BW. Therefore, only multi-component-based integrated disease management (IDM) can successfully control this disease. The use of organic amendments such as the addition of green manures and dried powders of the whole plant or plant parts to soil have been reported to be an effective component of the IDM against different diseases including BW (Naz *et al.,* 2015a and b; Din *et al.,* 2016). The addition of seed-cakes organic amendments of *Pongamia pinnata* and *Madhuca indica* to soil effectively controlled leaf spot (*Cercospora rauvolfiae*) of sarpagabdha (Arumugam *et al.,* 2010). Likewise, when infested soil was amended with *Brassica juncea* (L) or neem cakes (4 q/ha), rhizome yield was increased by four fold and soft rot (*Pectobacterium chrysanthemi*) Berkholder of *Aloe barbadensis* Miller and *Aloe vera* (L.) Tourn was reduced by 50% (Sharma *et al.,* 2010). Plants of Brassica species mulched during their flowering stage, release isothiocynates, nitriles and thiocynates. These plant chemicals are anti-bacterial and greatly reduce soil populations of *R. solanacearum* (Arthy *et al.,* 2005). Other plants such as Thymus spp. release volatile compounds (thymol in case of *Thymus* spp.) on their decomposition in soil which possess great anti-microbial effect against tomato wilt pathogen (Pradhanang *et al.,* 2003; Ji *et al.,* 2005; Ji *et al.,* 2007). Organic amendments have been reported (Qasem & Abu-Balan, 1996) to be effective, non-toxic and readily degradable. Moreover, the use of plant products to control diseases is economical, particularly if they are weeds, available in large amounts and free of cost. These qualities make such weeds an attractive component of IDM for the resource-poor farmers of developing countries like Pakistan.

Our study revealed that soil organic amendment with dried powder of *W. somnifera* applied at different times had a significant effect on the *In vitro* growth inhibition of the bacterium, improving plant growth characters *In vivo*, reducing bacterial load g^{-1} soil and affecting disease severity by decreasing the AUDPC values. Higher doses of leaves powder applied 20 days before transplanting tomato seedlings were found superior to the dried powder of other parts of the plant used at lower rates and applied fewer days before transplanting. Leaves of *W. somnifera*, reportedly (Singh & Kumar, 2011; Singh & Kumar 2012; Panchal *et al.,* 2016) contain higher amounts of anti-bacterial substances than the other parts of the plant. As compared to those of stems and roots, highest amounts of free and bound (10.5 mg/g and 3.5 mg/g dried plant part, respectively) flavonoids were found in leaves (Singh & Kumar, 2011). Although roots and stems of *W. somnifera* reportedly (Singh & Kumar, 2012) had more alkaloids (12 mg/g and 10.5 mg/g dried plant part, respectively), leaves were also found to contain significant amounts (7 mg/g dried plant part) of these plant bio-active compounds. Saponins and steroids were also reported to be present in significant amounts in leaves of *W. somnifera* (Panchal *et al.,* 2016). Flavonoids make complexes with extracellular and soluble proteins. They coagulate bacterial cell proteins as well as affect enzymes that synthesize some essential amino acids (Al-Obaidi, 2014). It has been reported that alkaloids inhibit important enzymes such as topoisomerase and damage DNA (Tanaka *et al.,* 2006), whereas steroidal saponins inhibit bacterial cell growth by damaging cell membrane through their reaction with membrane sterol (Wang *et al.,* 2000). This explains the superiority of leaves and succulent shoots (consisting of tender stems and leaves) over other parts of *W. somnifera* not only in producing as big zones of *In vitro* bacterial growth inhibition, using leaf aqueous extracts and agar well-diffusion assays, as produced by streptomycin but also in effectively controlling BW. Although organic solvent extracts of many plants are reportedly more effective than aqueous extracts (Jeyaseelan *et al.,* 2010) because of plant secondary metabolites being more soluble in organic solvents in restricting bacterial growth, aqueous extracts can be easily made, they are cheaper, and therefore, more affordable by our resource-poor local farmers.

The action of plant residue incorporated into soil is multipronged. The most obvious result of the decomposition of organic matter in soil is the release of anti-microbial substances, particularly at higher temperatures which enhance the release of such toxic substances (Bonanomi *et al.,* 2007). Interestingly, the communities of the anti-pathogenic soil microbes are neither negatively affected by these toxic substances (Klein *et al.,* 2011) nor the high temperatures (Porras *et al.,* 2007). These anti-bacterial compounds directly kill pathogens (Regnault-Roger *et al.,* 2005). Besides having the direct-action bactericidal chemicals, dried powders of plants contain a large number of compounds including some plant defense elicitors. These elicitor chemicals activate the inactive natural defenses of tomato plants (Kagale *et al.,* 2004; Walters *et al.,* 2005; Mitra & Paul, 2017). Hassan *et al.* (2009) demonstrated that the aqueous extracts of *Hibiscus sabdariffa*, *Punica granatum* and *Eucalyptus globulus* had both the ability to produce *In vitro* bacterial growth inhibition zones against potato bacterial wilt pathogen and the capacity to elicit systemic resistance in potato plants. The aqueous extracts of these plants applied to soil as drench, significantly reduced the severity, in comparison to inoculated control, of bacterial wilt of potato. Moreover, the activities of the defense-related enzymes such as peroxidase, polyphenoloxidase and phenyl alanine ammonia lyase were also significantly increased in extract-treated plants. This suggests the presence of both, the SAR eliciting natural compounds as well as the bactericidal compounds in the aqueous extracts of these plants. Consistent with the results of these authors, we also found bactericidal as well as SAR-eliciting activity in the aqueous extracts of *Eucalyptus globulus* (unpublished data). Kagale *et al.,* (2004) demonstrated that leaf extracts of *Datura metel* did both, restricted the *In vitro* bacterial growth as well as induced SAR against sheath blight and bacterial leaf blight disease of rice. SAR activation was evident from the enhanced levels of defense-related enzymes such as peroxidase, glucanase, chitinase, and PAL, whereas anti-microbial activity was shown by the *In vitro* bacterial growth inhibition zones. Although it was not determined which compound(s) were responsible for SAR activation, it was confirmed through mass spectrography that the compound that caused the *In vitro* growth inhibition of *Xanthomonas oryzae* pv. *oryzae* was a withanolide, called daturilin. Mitra & Paul, (2017) prepared a mixture of aqueous extracts (1:1 v/v) of tender core of *Musa acuminata* pseudostem (produced as agricultural waste after banana harvest) and *Tagetes erecta* leaves and found that the preparation had both anti-microbial and SAR-inducing properties. Using this mixture, the biochemical defense in several host plants including cucumber, barley, spinach and tomato, was sustained for several weeks. The researchers concluded that 1:10 dilution of the SAR-activating mixture was the most effective when sprayed before challenge inoculation. The improvement in soil physical structure and its characteristics as a result of addition of organic amendment is another well-known benefit. Soil water holding capacity, soil-ion adsorption capacity and soil pH buffering are reportedly improved by soil organic amendments (Braddy & Weil, 1999).

Our results that the effect of dried powders of *W. somnifera* against BW is dose-dependent corroborated the results of the earlier researchers. Using green manure (Naz, 2015a) or dried powder (Naz, 2015b) of different parts of *Fumaria parviflora*, Naz *et al.,* demonstrated that under both, green house and field conditions, nematode galls, GI, reproduction factor, egg masses and number of females per g of tomato (cv Rio Grande) roots correspondingly decreased when doses of organic amendment were increased. The higher dose of 30g dried powder of *F. parviflora* /kg potted soil controlled root knot nematodes as well as enhanced plant growth parameters more effectively than the lower doses. The researchers concluded that the higher doses of green manure organic amendment or dried powders of *F. parviflora* could be used alone or as an effective part of an integrated pest management strategy

against *Meloidogyne incognita*. The maximum safe dose of green manure organic amendment or dried powder, however, may vary from plant to plant and crop to crop depending on the nature of the plant used as organic amendment and the target crop to be protected against diseases. In an attempt to control *Sclerotium rolfsii* in onions, Flores-Moctezuma *et al.,* (2006) used naturally infested soil in micro plots amended with 50g/kg soil of dried powders of *Parthenium hysterophorus* in combination with solarization. The researcher found this treatment to be very effective in reducing the disease and sclerotial numbers of *S. rolfsii* with no noticeable symptoms of phytotoxic effect. Similarly, Cavoski *et al.,* (2012) used 60g/kg soil of green manure of *Melia azedarach* fruit to control *Meloidogyne incognita* in potted cucumber plants. This treatment acted as nematicide and reduced the nematode population in soil and in cucumber roots as well as activated the natural defences of cucumber plants. Even at this high dose, no phytotoxicity was observed. In our studies, the highest dose (45 g dried powder kg^{-1} soil) caused stunting of the treated plants suggesting phytotoxicity. This necessitates the determination of phytotoxic threshold level for each plant powder and each target crop before the commercial application of such powders.

Our results indicated that the application time of dried powders of *W. somnifera* 20 days before transplanting tomato seedlings was superior to other application times tested. These results could possibly be explained on the basis of more time available for the decomposition of the plant powders into toxic anti-microbial compounds and longer exposure of BW pathogen to such compounds. The later resulted in the reduction of the bacterial load g^{-1} soil which translated into lower disease severity in the treated plants and lower values of AUDPC. Results similar to ours were reported by Aliyu *et al.,* (2011). These researchers tested the influence of different rates and different times of application of neem leaf powder (applied to soil) on cucumber mosaic virus of cowpea. The researchers concluded that neem leaf powder at the rate of 0.125 kg/10 kg soil, applied two weeks before planting, significantly reduced disease severity in cowpea and enhanced plant growth parameters. We conclude that the treatment combination involving higher doses of dried powders of *W. somnifera* applied to soil 20 DBT has strong potential to be considered as an effective component of the IDM against BW.

There are several benefits of the use of dried powder of *W. somnifera* as soil organic amendment for the control of BW in tomato and possibly other crops. The plant is available year round, in large amounts and cost-free. Its dried powder has long shelf life and can be stored at room temperature for more than two years with no loss in anti-microbial properties (unpublished data). Moreover, because of cheap labor, the powder could be target-applied to individual tomato plants thus saving the in-put costs. Also, the powder could be easily transported (as compared to bulky plants) to those tomato-growing areas where the weed plant is not locally available. Although the plant is evergreen, various biotic and abiotic stresses and seasonal variations make the quality and required quantity of the weed plant uncertain. To avoid such circumstances, fresh and juicy new sprouts of the plant, easily available after spring rains, could be collected, dried and turned into powder. The dried powder formulation could even be made more effective by different treatments such as complete mechanical disruption of the dried tissue while making the powder, combining the pre-transplant powder application to soil with plastic-mulch solarization during hot summer days and combination of a small amount of some chemical with the dried powder. Powder particle size is important for its activity. Our preliminary results indicated that the *In vitro* bacterial growth inhibition zones of aqueous extracts prepared from very fine dried powder were significantly bigger than those produced by extracts prepared from relatively coarse particle powder (unpublished data). This suggests that more complete disruption of plant cells probably release more anti-microbial compounds resulting in bigger inhibition zones. Regarding pre-plant combination of soil solarization with dried powder organic amendment, it could be easily done by covering moistened powder-mixed soil with clear plastic sheets during hot summer days prior to tomato-growing season. We found this combination treatment to effectively reduce the bacterial loads g-1 soil (unpublished data). BW pathogen is reportedly (Kangkiattikajorn *et al.,* 2007) killed by soil temperatures of 45ºC or above for about two days. Additionally, plastic-mulch solarization of powder-mixed soils could enhance the putrefaction of the plant material and release volatile compounds (Bonanomi *et al.,* 2007) resulting in further declination of the soil-borne BW pathogen. The combination of small amounts of different chemicals could be tried to explore the possibility of further enhancement of the effectiveness of the dried powder against BW. As the dried powder consists of a large number of natural compounds, the possibility that the long term use of the dried powder could result in the development of resistance in BW pathogen is not very likely. However, long term studies in this regard would be worth-doing. In conclusion, only an elaborate IDM strategy including dried powders, green manures or plant extracts of *W. somnifera* or other plants as effective components or SAR activators, in addition to the use of other approved components, will be able to fight off this multifaceted pathogen.

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