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RESEARCH ARTICLE

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Phytobiocidal management of bacterial wilt of tomato caused by *Ralstonia solanacearum* (Smith) Yabuuchi

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Abstract

Phytobiocides are a good alternative to chemicals in managing bacterial diseases including bacterial wilt of tomato caused by *Ralstonia solanacearum.* In the present research study, finely ground dried powders of seven widely available medicinal plants/ weeds species *viz., Peganum harmala* (esfand or wild rue), *Calotropis procera* (sodom apple), *Melia azedarach* (white cedar), *Allium sativum* (garlic), *Adhatoda vasica* (malabar nut), *Tagetes patula* (marigold) and *Nerium oleander* (oleander) were assessed for their anti-microbial activity, both *in-vitro* (10% w/v) and *in-vivo* (10, 20, 30, and 40 g/kg of potted soil) against *R. solanacearum.* Aqueous extracts (prepared as 10% w/v, soaking for 48-72 h and filtering) of *C. procera, A. vasica*, and *T. patula* inhibited the *in-vitro* growth of the bacterial pathogen over 60% of that produced by the standard antibiotic streptomycin. *A. sativum*, *N. oleander* and *P. harmala* aqueous extracts were less effective while *M. azedarach* showed no effect against *R. solanacearum.* The higher dose (40 g/ kg of soil) of *C. procera, A. vasica* and *T. patula* decreased disease severity quite effectively and increased yield and plant growth characters as much as the standard antibiotic did. No phytotoxicity of medicinal plants powder was observed on tomato plants. Alkaloids, flavonoids, tannins, saponins and terpenoids were detected in the aqueous extracts of *T. patula* and *A. vasica* whereas *C. procera* was found to have only alkaloids, flavonoids, tannins and saponins. Our data suggest that dried powders of *T. patula, C. procera* and *A. vasica* (40 g/kg of soil) could be used as an effective component in the integrated disease management programs against bacterial wilt of tomato.

Additional key words: *Peganum harmala*; *Calotropis procera*; *Melia azedarach*; *Allium sativum*; *Adhatoda vasica*; *Tagetes patula; Nerium oleander.*

Abbreviations used: ASM (acibenzolar-S-methyl); BW (bacterial wilt); Cmm (*Clavibacter michganensis* subsp. *michiganensis*); CRD (completely randomized design); LSD (least significant difference); NA (nutrient agar); Pst (*Pseudomonas syringae* pv. *tomato*); SDW (sterilized distilled water); UOA (University of Agriculture); Xv (*Xanthomonas vesicatoria*).

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Introduction

Tomato (*Solanum lycopersicum* L.), is an important vegetable crop throughout the world. In Pakistan, tomato is cultivated on 52,000 hectares with an annual production of 529,600 tons (Anonymous, 2011). To meet the consumer's demand, the country frequently imports tomatoes from India and Afghanistan. Bacterial wilt of tomatoes (BW), caused by *Ralstonia solanacearum*, is one of the most destructive diseases of tomatoes (Sharma & Kumar, 2004; Allen *et al*., 2005; Kang *et al*., 2007; Chaudhry & Rashid, 2011). Depending upon host, cultivar, climate, soil type, cropping pattern and the particular strain of the bacterium, *R. solanacearum* has been reported to cause direct yield losses of 0-91% in tomato, 33-90% in potato, 10-30% in tobacco, 80-100% in banana, and 0-20% in groundnuts (Elphinstone, 2005). The disease is more prevalent in tropical and sub-tropical regions where the environmental conditions (high humidity and temperature) are more favorable for disease development. BW has recently been reported from Pakistan. The incidence of

the disease was found to be 21.9% on sweet peppers, 16.6% on hot peppers, 13.3% on tomatoes, 10.5% on potatoes and 5.5% on brinjals. Over 80% of *R. solanacearum* strains present in Pakistan belong to biovar 3 (Begum *et al*., 2012; Shahbaz *et al*., 2015).

BW, a hard-to-control disease, cannot be sustainably managed by any single control method. The best management strategy is to integrate different control methods including the use of natural host–plant resistance, cropping systems (Dalal *et al*., 1999), genetically modified resistant plants (Shi-Rong *et al*., 1999), biological control (Katayama & Kimura, 1987), limited use of chemicals, and soil amendments (Michel & Mew, 1998). Chemicals, however, are hazardous to environment and cause the development of chemical-resistant pathogens. Therefore, there is a dire need to replace or at least reduce the amount of chemicals and to safeguard the environment. To achieve this goal, various non-pesticide control measures have been investigated by different researchers with varying degrees of success in controlling BW. Green manure of above-ground parts of pigeon pea (*Cajanus cajan*) and crotalaria (*Crotalaria juncea*) completely suppressed tomato BW in 45 days (Cardoso *et al*., 2006). Field application of thymol oil (derived from thyme plant) at the rate of 0.72% reduced BW of tomato by 65-82% (Ji *et al*., 2005). Combination of acibenzolar-S-methyl (ASM) and thymol significantly reduces the incidence of BW and increased yield of tomato (Hong *et al*., 2011). ASM induces systemic resistance in treated plants (Pradhanang *et al*., 2005; Hacisalihoglu *et al*., 2007). Paret *et al*., (2010) evaluated the effect of essential oils derived from palmarosa (*Cymbopogon martini*), lemongrass (*C. citratus*) and eucalyptus (*Eucalyptus globulus*) on *R. solanacearum* race 4 and BW of edible ginger. Palmarosa and lemongrass oils, at concentrations of 0.07 and 0.14%, caused complete inhibition of the pathogen growth in culture amendment assays, reduced the bacterial numbers to undetectable levels in infested potting medium, and significantly reduced BW incidence.

Use of plant-based natural products is one of the effective alternatives to chemicals for controlling plant diseases including BW. Plant products are safe, non-phytotoxic, systemic and biodegradable (Tripathi & Dubay, 2004). Plant products can be used as green manures, dried powders (Naz *et al.*, 2015a,b) and aqueous or organic solvent extracts (Yesmin *et al*, 2008; Balestra *et al.*, 2009). Some plants, including medicinal plants, have larger amounts of anti-microbial secondary metabolites. When dried powders of such plants are used as soil organic amendments, the powders get mixed with soil water, get decomposed and release water-soluble anti-microbial secondary metabolites which protect host plants against pathogens (Naz *et al*., 2015b). The aim of this study was to explore the anti-microbial potential of those traditional medicinal plant species which grow wildly, are easily available in large numbers and free of cost. Seven such plant species viz *Peganum harmala* (esfand or wild rue), *Calotropis procera* (sodom apple), *Melia azedarach* (white cedar), *Allium sativum* (garlic), *Adhatoda vasica* (malabar nut), *Tagetes patula* (marigold) and *Nerium oleander* (oleander) were selected for the study.

P. harmala, a perennial, bushy and wild-growing flowering plant is widely distributed in North Africa, Middle East, Turkey, Iran, India and Pakistan (Duran & Hamzaoglu, 2002; Ehsanpur & Sadat, 2002; Yousefi *et al*., 2009). It is rich in medicinal alkaloids including harmaline, harmine, harmalol and peganine (Massoud *et al*., 2002; Lala *et al*., 2004; Astulla *et al*., 2008; Bukhari *et al*., 2008). *C. procera*, a drought-resistant, salt-tolerant, small, erect and compact shrub weed is native to India, Pakistan, Nepal, Afghanistan, Iran, Middle East, and many parts of Africa. This shrub, well-known for its medicinal properties (Murti *et al*., 2010), is used in several traditional medicines and is known to possess analgesic, antitumor, antioxidant, hepato-protective, anticonvulsant, antimalarial and antimicrobial activity (Sharma *et al*., 2011). Using an agar well diffusion method, methanol and aqueous extracts of *C. procera* leaves were shown to be phytobiocidal to both gram-positive and gram-negative bacteria. Both extracts produced clear zones of inhibition as compared to the standard antibiotic streptomycin (Yesmin *et al*., 2008). *M. azedarach*, a deciduous, shade, ornamental and medicinal tree, is native to India, Pakistan, Iran and China. It has been naturalized in several regions of the world (Nakatani *et al*., 1998). *A. sativum*, a bulbous herb and native to Middle Asia (Petrovska & Cekovska, 2010) has tremendous traditional dietary and medicinal applications (Ross *et al*., 2001). Balestra *et al*. (2009) sprayed aqueous extracts of fresh *A. sativum* (1%, w/v) and *Ficus carica* (30%, w/v) on 1-month-old potted tomato plants (cv. Pullrex) 24 h before their inoculation with tomato bacterial pathogens *i.e*., *Pseudomonas syringae* pv. *tomato* (Pst), *Xanthomonas vesicatoria* (Xv) and *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), causal agents of bacterial speck, bacterial spot and bacterial canker, respectively and found that the extracts were effective in reducing disease incidence and disease severity. These plant extracts resulted in effective disease control of up to 65% (*A. sativum*) and 38% (*F. carica*) of that of the standard copper treatment. The researchers concluded that greenhouse-grown-tomatoes could be protected against Pst, Xv and Cmm by using the aque-

ous extracts of *A. sativum* and *F. carica*. *A. vasica*, indigenous to India, is an evergreen shrub and is popularly used for the treatment of cough, asthma, colds, rheumatic diseases and traumatic injuries (Gao *et al*., 2008). In Pakistan, this plant grows wildly, in large quantities, in mountains, waste places and along road sides and is available free of cost. Aqueous and methanolic extracts of *A. vasica* were found to have strong anti-oxidant and anti-microbial activity against some human bacterial pathogens (Kaur *et al*., 2012). *T. patula,* native to North and South America, includes annual and perennial, wild and cultivated herbaceous plants which have been shown to possess anti-microbial, antifungal and antiviral activities (Soule, 1996; Russel *et al*., 1997). *N. oleander*, an evergreen shrub, grows in the Mediterranean, Syria, Turkey (Bas *et al*., 2012), India and Pakistan. This plant has been used as anti-microbial, anti-leprotic, anti-cancer, cardiotonic and central nervous system depressant (http://www.inchem.org/documents/pims/plant/pim366.htm).

Keeping in view the economic importance of tomatoes in Pakistan, the losses caused by BW, lack of resources among farming communities, the cost-free and easy availability of these medicinal plants, we conducted both *in-vitro* and *in-vivo* studies investigating the potential of these plant species to be used as an eco-friendly and effective component in the integrated management against bacterial wilt of tomato.

Material and methods

Bacterial culture and inoculum production

R. solanacearum was obtained from the plant bacterial culture bank of the Department of Plant Pathology, the University of Agriculture (UOA), Peshawar. The strains of the bacterium used in this study belonged to the most aggressive biovar, *i.e*., biovar 3 (unpublished data). The bacterium was sub-cultured on nutrient agar (NA) medium (per 1 L: beef extract, 3 g; peptone, 5 g; agar, 15 g) at 28 °C for 48 h (Ramesh *et al*., 2009). The bacterial cells were flooded with sterilized distilled water (SDW) and scrapped off the NA plate with sterilized cotton swab to make a bacterial suspension (Wai *et al*., 2013). The concentration of the suspension was adjusted to 1.5×10^8 cfu/mL (OD_{600} = 0.3) in a spectrophotometer (Hindi, 2013; Lin *et al*., 2014). This suspension was used for *in-vitro* bacterial inhibition tests as well as for inoculation of healthy plants during different *in-planta* experiments. Morphological characteristics such as colony color, colony shape, margins, elevation and texture were studied to confirm the purity of the culture.

Pathogenicity tests

Pathogenicity of *R. solanacearum* was confirmed by inoculating 4-weeks old tomato plants and tomato fruits with a bacterial suspension (1.5×10^8 cfu/mL). The plants were kept in growth chamber (average RH = 80%, temperature, 25-30 °C, dim light for 12 h daily) for up to two weeks while fruits were kept in incubator at 28 °C. Typical symptoms of the disease were noticed on plants and tomato fruits. The bacterium was re-isolated from stem and fruit sections by crushing them in sulphate buffer and plating on nutrient agar plate (EU, 1998).

Medicinal plant species and their extracts

Green leaves and succulent branches of seven medicinal species (10-40 individual plants per species) viz *P. harmala, C. procera, M. azedarach, A. sativum, A. vasica, T. patula* and *N. oleander* were collected from different regions of Mohmand and Khyber Agencies, Peshawar, Pakistan, authenticated by a weed botanist, and specimens were deposited in the herbarium of UOA, Peshawar, Pakistan. Plant materials of each species were pooled together, washed with tap water, cut into small pieces, shade-dried (Mahlo *et al*., 2010) for 45 days and ground to make fine powder (2 mm mesh) with the help of a grinder (Kumar *et al*., 2011). The powder of each plant was stored separately at 4 °C (Oguwike *et al*., 2013). To obtain plant aqueous extracts, the ground powder of each plant was separately soaked in water for 48-72h (Frey and Meyers, 2010) by mixing 10g powder with SDW and adjusting the volume to 100 mL (10% w/v). The suspension was filtered through Whatman filter paper (20 µm) to remove large plant particles. This filtrate was used in different dilutions (0% or undiluted, 25%, 50%, and 75%).

In-vitro studies

The different dilutions of aqueous extracts of the medicinal plants were tested for their ability to inhibit the *in-vitro* growth of *R. solanacearum* using paper disc (6 mm, Whatman) diffusion method (Bauer *et al.,* 1996). A bacterial lawn was prepared by pouring 100 µL of the bacterial suspension (1.5×10^8 cfu/mL) on NA plates and spreading uniformly (Balestra *et al*., 2009; Hindi *et al*., 2013). Paper discs were loaded with 20 µL of each plant extract dilution and air-dried. Negative and positive control paper discs were loaded with 20 µL of SDW and streptomycin (200 ppm) re-

spectively. Each plate received six paper discs; four discs of different dilutions, one disc of standard antibiotic streptomycin and one disc of SDW (Frey & Meyers, 2010). The plates were incubated overnight at 30 °C and the zones of inhibition were measured across the discs with transparent plastic ruler. The experiment was done three times using completely randomized design (CRD) with five replications.

Detection of secondary metabolites in aqueous extracts of plants

Based on their performance in *in-vitro* tests against *R. solanacearum,* aqueous extracts (10% w/v, prepared as described before) of some plants were tested for the presence of major anti-microbial plant secondary metabolites in them. Alkaloids were detected by mixing 1.5 mL of 1% HCl with 2 mL of plant aqueous extract, heating the mixture in water bath for a few minutes and adding 6 drops of Mayer's reagent. Appearance of orange precipitate was indicative of alkaloids (Rasool *et al*., 2010). For the detection of terpenoids, the protocol of Vijay *et al*. (2013) was followed. For this purpose, 2 mL of aqueous extract were mixed with 2 mL of chloroform, the mixture was evaporated and the resulting fine powder was mixed with 2 mL of concentrated sulphuric acid. The mixture was heated for 2 min. Formation of greyish color indicated the presence of terpenoids. To detect flavonoids, a few drops of lead acetate were mixed with 2 mL of plant aqueous extract. Formation of yellow precipitate was considered as the presence of flavonoids (Salhan *et al*., 2011). To confirm the presence of tannins and saponins in the aqueous extracts of plants, the protocol of Saidulu *et al*. (2014) was used. Tannins were considered to be present if blackish precipitate appeared after mixing a few drops of ferric chloride with 5 mL of the aqueous extract. Similarly, saponins were considered to be present if frothing appeared and persisted after adding 2 mL of plant extract to 5 mL of distilled water and agitated for 3 min.

In-vivo studies

Experiments were conducted in screen house to study the effect of dry powders of five selected plant species (*C. procera, A. sativum, A. vasica, T. patula,* and *N. oleander*) on the control of bacterial wilt of tomatoes. Tomatoes (cv. Rio Grande) were cultivated in large earthen pans filled with heat-sterilized soil (100 °C for 6 h). One-month old plants were transplanted (1 seedling/pot) to 15 cm-diameter earthen pots (Ramesh *et al*., 2009). Each pot had 2 kg of pasteurized mixture of clay and sand (2:1 w/w). Different doses, *i.e*., 10, 20, 30 and 40 g/kg of soil (Flores-Moctezuma *et al*., 2006) of dry powder of the medicinal plants were mixed with soil before transplanting seedlings. Plants were inoculated by clipping the lower leaf with scissor dipped in fresh bacterial suspension of 1.5×10^8 cfu/mL (Hindi, 2013). For comparison, standard antibiotic, streptomycin (200 ppm, 30 mL/pot, applied as drench 4 times at 20 days interval) and control treatment (inoculated with bacteria, no treatment applied) were also included in the experiment. CRD with five replications was used for the experiment. The potted plants were watered as needed and fertilized once with 100 mL/plant of 0.1%, 25-5-32 + micronutrients hydro-sol fertilizer (Engro Crop Ltd.) one week after transplanting (Kokalis-Burelle *et al*., 2005). The experiment was terminated 90 days after transplanting and the data were recorded on disease severity, plant height (cm), tomato yield/plant (g), plant fresh biomass (g), number of shoots/plant, root weight (g), and plant dry biomass (g).

Disease severity (%)

The 1-5 disease severity scale of Wai *et al*. (2013) based on the degree of drooping and wilting of leaves, was slightly modified (to make it simpler while retaining its usefulness) and used for recording disease severity data. The modifications were that the five category scale was reduced to four categories by merging together categories 3 and 4 of the original scale. The various categories of the modified scale are, 1 = no visible symptoms, 2 = one leaf to half of the foliage wilting, 3 = nearly all of the foliage wilting, 4 = the whole plant wilting and dead. Disease severity (%) for each treatment was then computed using the formula developed by Bdliya & Dahiru (2006), $S = 100\sum n/4N$, where S = disease severity (%), $\sum n$ = summation of individual ratings, 4 is the highest category on the rating scale and N = total number of plants per treatment. Four severity data points were recorded for each treatment with two weeks interval.

Data analysis

Data recorded for disease severity, yield and plant growth characters under *in-vitro* and *in-vivo* conditions were considered as dependent factors, while different treatments (different medicinal plant extracts and their doses) were considered as independent factors using CRD. The data were subjected to analysis of variance using Statistix (NH Analytical Software, Roseville, MN, USA) (Campbell & Madden, 1990). Treatment

means of the different dependent factors were compared using Fisher's protected least significance difference (LSD) test at p=0.05 (Gomez & Gomez, 1984).

Results

Morphology and pathogenicity of *R. solanacearum*

To verify that the stored cultures remained pure and did not lose pathogenicity during storage, its morphological characteristics were observed and pathogenicity was tested before using it for different experiments. On nutrient agar, the bacteria produced off-white/cream colored, irregular, fluidal and non-transparent colonies after 48 hours of incubation at 28 °C. Plants inoculated with *R. solanacearum* showed symptoms of bacterial wilt disease 8 days after inoculation. The first symptoms appeared as wilting of the youngest leaves or leaflets. Later on, branches also showed wilt symptoms. The bacteria were re-isolated from the infected plant samples to confirm Koch postulates.

The *in-vitro* anti-microbial activity of aqueous extracts of the medicinal plants

C. procera, A. vasica, T. patula and *A. sativum* were found to have anti-microbial activity against the bacterial pathogen in agar plate diffusion assay. All dilutions of *M. azedarach, P. harmala* (except 0% dilution), *N. oleander* (except the 0% dilution) and the lowest dilution (75%) of *A. sativum* and *T. patula* did not inhibit the *in-vitro* growth of the bacterium. The un-diluted (0% dilution) aqueous extract of *C. procera* produced the maximum (13 mm) zone of inhibition, followed by that of *A. vasica* (12.20 mm) and *T. patula* (12 mm) (Table 1 and Fig. 1). The results of the inhibition zones produced by the undiluted and 25% diluted aqueous extracts of *C. procera* were statistically at par with those produced by the undiluted extracts of *A. vasica* and *T. patula*. In comparison with the standard antibiotic streptomycin (200 ppm), the undiluted extracts of *C. procera*, *A. vasica* and *T. patula* were 63%, 59% and 58%, respectively, as effective as the antibiotic.

Detection of secondary metabolites in aqueous extracts of plants

To investigate if there was any correlation between the presence or absence of a certain secondary metabolite in the aqueous extract of a medicinal plant and its ability to inhabit the *in-vitro* bacterial growth, the extracts of five plant species (*T. patula, A. vasica, C. procera*, *A. sativum* and *N. oleander*) were used for the detection of these metabolites. The results (Table 2) revealed the presence of all major anti-microbial secondary metabolites like alkaloids, flavonoids, tannins, saponins and terpenoids in the aqueous extracts of *T. patula, A. vasica*, *C. procera* (terpenoids, undetectable) and *N. oleander* (saponins, undetectable). However, *A. sativum* lacked alkaloids, flavonoids and tannins.

In-vivo studies

Effect of medicinal plant's powder on BW severity

To investigate whether the *in-vitro* anti-microbial activity of the medicinal plant species could result

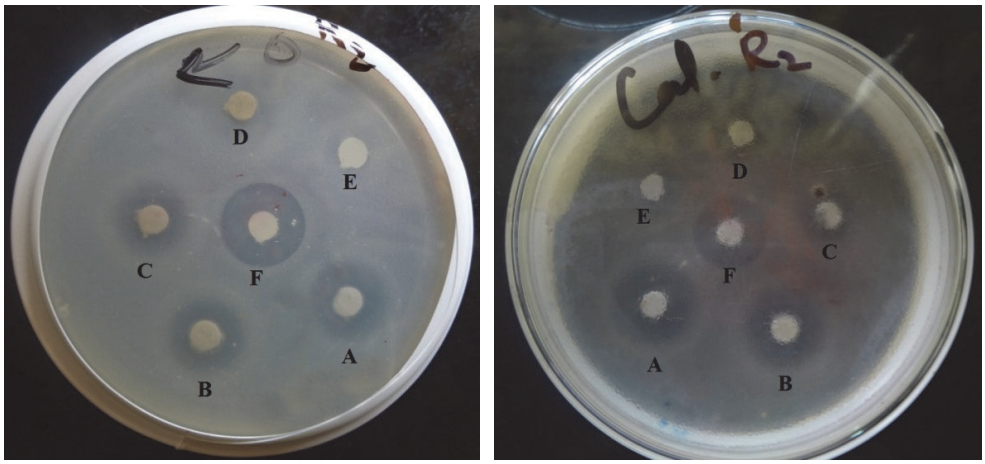

Figure 1. Zones of inhibition produced by *A. vasica* (left) and *C. procera* (right) aqueous extracts, in the following dilutions: A, undiluted or 0%, B, 25%, C, 50%, D, 75%. E, water control, F, antibiotic streptomycin.

Table 1. Effect of medicinal plant aqueous extracts and their dilutions on the *in-vitro* growth inhibition of *R. solanacearum* 24 hours after incubation at 30 °C

Plant	Concentration	Inhibition zone (mm)	% Decrease over streptomycin
M. azedarach	100%	0.00 j	-
M. azedarach	50%	0.00 j	-
M. azedarach	33%	0.00 j	-
M. azedarach	25%	0.00 j	-
N. oleander	100%	9.20 ef	55.34
N. oleander	50%	0.00 j	-
N. oleander	33%	0.00 j	-
N. oleander	25%	0.00 j	-
A. sativum	100%	9.40 de	54.37
A. sativum	50%	8.60 efg	58.25
A. sativum	33%	7.40 gh	64.08
A. sativum	25%	0.00 j	-
C. procera	100%	13.00 b	36.89
C. procera	50%	11.20 c	45.63
C. procera	33%	9.60 de	53.40
C. procera	25%	8.20 efgh	60.19
T. patula	100%	12.00 bc	41.75
T. patula	50%	8.60 efg	58.25
T. patula	33%	7.80 fgh	62.14
T. patula	25%	0.00 j	-
A. vasica	100%	12.20 bc	40.78
A. vasica	50%	10.80 cd	47.57
A. vasica	33%	9.00 ef	56.31
A. vasica	25%	7.00 h	66.02
P. harmala	100%	2.80 i	86.41
P. harmala	50%	0.00 j	-
P. harmala	33%	0.00 j	-
P. harmala	25%	0.00 j	-
Streptomycin	200 ppm	20.60 a	-
Control (water)		0.00 j	-
LSD = 1.40			

The values shown are the mean of five replicates. Means within column followed by different letters are significantly different from each other ($p \leq 0.05$).

into their ability to control BW *in-planta*, four powder doses (10, 20, 30, and 40 g/kg soil), of each plant species (except *P. harmala* and *M. azedarach* which showed very little and no *in-vitro* anti-microbial activity, respectively) were tested for the control of the disease. All doses (except 10 g/kg soil) of ground dry powders of all tested plant species significantly ($p < 0.05$) reduced disease severity at 14-, 28-, 42- and 56-days post-inoculation as compared to control (Table 3). However, in comparison with the disease severity of the antibiotic-treated plants at 56-days post inoculation, only the higher dose (40 g/kg soil) of *T. patula, A. vasica and C. procera* performed better. Plants treated with 40 g kg^{-1} soil of *C. procera, T. patula* and *A. vasica* had 31.17%, 33% and 39.50% respectively more disease severity than those treated with the antibiotic. Disease severity levels of plants treated with *C. procera, T. patula* and *A. vasica* were statistically at par with each other. Tomato plants treated with dry powder doses of 40 g/kg of soil showed no phytotoxic effects (N. Din, *pers. observ*.).

Table 2. Detection of secondary metabolites in aqueous extracts of medicinal plants

Medicinal plants	Secondary metabolites				
	Alkaloids	**Flavonoids**	**Tannins**	**Saponins**	**Terpenoids**
T. patula	+	+	+	+	+
A. vasica	+	+	+	+	+
C. procera	+	+	+	+	-
A. sativum	-	-	-	+	+
N. oleander	+	+	+	-	+

+ indicates presence and – indicates absence of chemical.

Table 3. Effect of medicinal plants powder and their doses on disease severity 14, 28, 42 and 56 days post-inoculation on *R. solanacearum*-inoculated tomato plants

Plant	Dose (g/kg soil)	14 days post-inoculation	28 days post-inoculation	42 days post-inoculation	56 days post-inoculation
T. patula	10	56.26 b	47.76 bcd	37.73 cde	34.33 cd
T. patula	20	46.00 def	34.93 hi	30.167 fgh	26.46 fg
T. patula	30	38.33 ghi	25.66 jk	23.83 ij	22.66 hi
T. patula	40	30.44 jk	24.66 k	23.33 ij	18.66 j (33.00)
A. vasica	10	54.50 b	46.33 cde	39.16 c	35.76 c
A. vasica	20	47.00 de	36.00 ghi	30.00 fgh	28.33 ef
A. vasica	30	37.33 hi	25.00 jk	23.33 ij	23.33 ghi
A. vasica	40	28.80 kl	23.66 k	21.50 j	20.66 ij (39.50)
C. procera	10	46.16 def	41.66 efg	39.00 cd	26.50 fg
C. procera	20	38.00 ghi	35.46 hi	31.56 gh	22.50 hi
C. procera	30	34.00 ij	23.33 kl	20.00 jk	18.83 j
C. procera	40	25.66 l	21.66 kl	19.16 jk	18.16 j (31.17)
N. oleander	10	55.33 b	44.66 cdef	38.83 cd	35.83 c
N. oleander	20	50.00 cd	46.10 cde	34.33 def	28.66 ef
N. oleander	30	44.66 ef	40.66 efgh	33.83 ef	26.00 fgh
N. oleander	40	39.33 gh	35.16 hi	29.00 gh	24.00 ghi
A. sativum	10	53.66 bc	52.800 b	50.66 b	41.66 b
A. sativum	20	61.50 a	49.00 bc	47.33 b	31.86 de
A. sativum	30	46.90 de	42.36 def	33.33 efg	26.60 fg
A. sativum	40	42.00 fg	39.53 fgh	32.66 fg	25.33 fgh
Streptomycin	200 ppm	30.66 jk	21.33 kl	16.00 kl	12.50 k
Control (Water)		53.67 bc	64.66 a	72.00 a	82.83 a
LSD		4.38	6.05	4.71	3.63

Values are the mean of five replicates. Means within a column with different letters are significantly different ($p \leq 0.05$). Numbers in parenthesis show % increase over antibiotic.

Effect of medicinal plant's powder on tomato yield and plant growth characters

The effect of the dry powder of the medicinal plant species *C. procera, T. patula* and *A. vasica* on the yield and plant growth characters of the inoculated tomato plants followed almost similar pattern as observed in case of disease severity. Higher yields, shoot and root lengths and fresh and dry biomasses were obtained in case of plants treated with the higher dose (40 g/kg soil) of *C. procera, T. patula* and *A. vasica* (Table 4). Mean yield (except that of 40 g/kg soil of *T. patula*-treated plants), plant height, fresh and dry biomasses and mean number of shoots/plant (except those of 40g/kg soil of *T. patula*-treated and *A. vasica*-treated plants) of plants treated with the higher dose (40 g/kg soil) of *C. procera*, *T. patula* and *A. vasica* were statistically at par with those of antibiotic-treated plants. However, values of yield and plant growth characters obtained with almost all doses of *A. sativum* and *N. oleander* were statistically not different from those of the control.

Discussion

Green plants are major reservoirs of anti-microbial bioactive compounds and therefore can be used as source for the development of natural pesticides (Jeyaseelan *et al*., 2010). Many plants have been found to be effective against bacteria (Ranjit *et al*., 2012), fungi

Table 4. Effect of medicinal plants powder on yield and plant growth characters of tomato plants inoculated with *R. solanacearum*.

Plant	Dose (g/kg soil)	Yield/plant (g)	Plant height (cm)	Fresh biomass (g)	Dry biomass (g)	Shoots/plant
T. patula	10	413.33 fgh	53.00 ij	101.67 ef	22.33 fg	4.33 h
T. patula	20	292.00 kl	56.00 hij	110.00 ef	26.33 efg	5.00 gh
T. patula	30	524.00 de	69.33 ef	114.33 ef	28.33 efg	5.33 fgh
T. patula	40	753.67 b	90.00 bcd	417.33 ab	113.83 b	9.33 cd
		(-17.84)	(-3.92)	(-0.95)	(-5.67)	(-9.68)
A. vasica	10	392.00 ghij	57.33 ghij	109.33 ef	24.00 fg	5.00 gh
A. vasica	20	620.00 cd	59.33 ghi	112.17 ef	27.66 efg	5.00 gh
A. vasica	30	650.00 c	83.00 cd	279.67 c	85.00 c	7.00 e
A. vasica	40	826.67 ab	92.00 abc	386.33 b	117.67 b	9.00 d
		(-9.88)	(-1.78)	(-8.33)	(-2.49)	(-12.88)
C. procera	10	342.33 ghijk	57.00 ghij	105.00 ef	23.33 fg	4.33 h
C. procera	20	418.33 fgh	58.66 ghij	111.17 ef	29.66 efg	5.33 fgh
C. procera	30	880.33 a	82.66 d	403.67 b	113.00 b	8.66 d
C. procera	40	886.67 a	93.67 ab	420.67 ab	118.67 b	10.66 b
		(-3.34)	(0.00)	(-0.16)	(-1.66)	(+3.19)
N. oleander	10	399.33 ghi	54.66 ij	100.00 f	21.00 g	4.33 h
N. oleander	20	398.00 hi	58.33 ghij	106.00 ef	23.33 fg	5.33 fgh
N. oleander	30	272.67 kl	60.33 fghi	117.67 ef	26.33 fg	5.33 fgh
N. oleander	40	515.33 ef	71.66 e	152.67 e	33.167 e	6.33 ef
A. sativum	10	265.00 kl	50.00 j	90.33 f	26.00 efg	3.00 i
A. sativum	20	299.00 ijkl	56.00 hij	100.67 f	27.33 efg	4.33 h
A. sativum	30	322.33 hijk	59.66 ghi	109.67 ef	30.667 ef	5.66 fg
A. sativum	40	435.33 efg	66.00 efg	122.17 ef	32.833 e	5.66 fg
Streptomycin	200 ppm	917.33 a	93.667 ab	421.33 ab	120.67 ab	10.33 bc
Control (water)		207.6 l	54.66 ij	120.17 ef	25.00 efg	4.33 h
LSD		102.69	9.09	51.34	8.79	1.07

Values are the mean of five replicates. Means within a column with different letters are significantly different ($p \leq 0.05$). Numbers in parenthesis show % change over antibiotic (negative sign means decrease) and numbers with positive sign show % increase over antibiotic.

(Kareem *et al*., 2008), nematodes (Al-Shaibani *et al*., 2008), protozoa and some viruses (Khan *et al*., 2001). Because of being environment friendly and effective against plant pathogens, dried powders/green manures of various plants could be added to soil as organic amendments (Naz *et al*., 2015a,b) or soil could be drenched with plant aqueous extracts (Deberdt *et al*., 2012) to replace or reduce the amount of chemicals used for the control of plant diseases.

The *in-vitro* growth inhibition of *R. solanacearum* by the undiluted aqueous extracts of *C. procera, A. vasica T. patula,* and *A. sativum* may be explained on the basis of the presence and amounts of anti-microbial secondary metabolites in these plants. Some plant extracts were more effective than others. It may be due to the higher amounts of some secondary metabolites present in the plant or they may contain some other compounds which are responsible for anti-microbial properties and yet not identified. Using agar well diffusion assay, Jeyaseelan *et al*. (2010) found out that both organic solvents and aqueous extracts of *A. sativum* bulbs restricted the *in-vitro* growth of plant pathogenic bacteria *i.e*., *X. axonopodis* and *R. solanacearum*. They also found the organic solvent (ethyl acetate) extracts to be twice as effective as aqueous extracts against both types of bacterial pathogens indicating more solubility of anti-microbial chemicals of garlic in organic solvents. Aqueous extracts, however, are cheap and easy to make and more affordable for the resource-poor farmers of developing countries. Consistent with our results, these researchers were not able to find alkaloids, flavonoids and tannins in the aqueous extracts of garlic bulbs but detected them when ethyl acetate was used as solvent. Instead of performing frothing test (Saidulu *et al*., 2014), Al-Obaidi (2014) used mercuric chloride as reagent, but was unable to detect saponins in the aqueous extracts of *N. oleander*; however, he found that the aqueous extract of *N. oleander,* contrary to our findings, effectively inhibited the *in-vitro* growth of *Staphylococcus aureus* and *Escherichia coli*. Larger growth inhibition zones were recorded for higher concentrations of *N. oleander*. This difference in results might be due to different nature of the bacterial pathogens tested as well as the concentration of the extract used. Consistent with this notion, we found some growth inhibition of *R. solanacearum* when

undiluted extract was used. Because of being available in large quantities and free of cost, *C. procera, A. vasica* and *T. patula* are better choices than *A. sativum* for controlling BW.

The presence of bioactive compounds is known to exhibit medicinal as well as physiological activities (Iraqui *et al*., 2013). Flavonoids are phenolic compounds and are reported to be synthesized by the plant in response of pathogen attack. They have the ability to make complexes with extracellular and soluble proteins. Flavonoids have also been reported to coagulate bacterial cell proteins and to affect enzymes involved in synthesis of essential amino acids (Al-Obaidi, 2014). Tannins have anti-microbial properties due to their basic character. Tannins react with prolin-rich proteins and form stable water soluble compounds. They also kill bacteria by directly damaging their cell membrane (Mainasara *et al*., 2012). Tannins bind to adhesins so that the bacteria cannot attach to the surface of the host and in this way they remain unable to cause infection (Wang, 2014). Saponin is a broad group and has been classified into various categories on the basis of its function. Steroidal saponins are reported to inhibit bacterial growth, because they react with membrane sterol and stop membrane function which leads to inhibition of cell growth (Wang *et al*., 2000). Alkaloids are also a vast group. Some alkaloids inhibit important enzymes such as topoisomerase. Other alkaloids such as bisindole monoterpenoid alkaloids, act as DNA intercalating agent which sometimes result in certain kind of poisoning (Tanaka *et al*., 2006). Terpenoids are reported to inhibit bacterial growth by denaturation of proteins or by acting as dehydrating agents. Terpenoids act upon the phospholipid bilayers of the cell, due to which different processes like electron transport, protein translocation, phosphorylation steps and other enzyme-dependent reactions are affected and finally membrane disruption occurs which results in bacterial growth inhibition (Dorman & Deans, 2000).

Three different mechanisms might be responsible for the observed lower disease severity of the inoculated tomato plants treated with 40 g/kg soil of *C. procera, A. vasica* and *T. patula.* The most obvious one is the presence of various anti-microbial compounds in the dried powders added to soil. These compounds, when present in optimum concentrations, will directly kill the pathogen (Regnault-Roger *et al*., 2005). Our results indicated that all major anti-microbial compounds (*i.e*., alkaloids, flavonoids, tannins, saponins and terpenoids) were present in the dried powders of *C. procera* (except terpenoids), *A. vasica* and *T. patula.* Stimulation of antagonistic or competitive microorganisms, as a result of addition of dried powders of the medicinal plants to soil, could be another reason for the lower disease severity of the treated plants. This hypothesis, however, needs to be critically tested by determining the changes in the level of soil microbes before and after the addition of these powders, using appropriate protocols such as analysis of phospholipid fatty acids (PLFAs). Analysis of changes occurring in soil microbial populations using PLFA analysis provides direct information for the identification, classification and quantification of microbial community composition which overcomes the selectivity problems associated with culture techniques (Dong *et al*., 2014). The third mechanism could be the presence of natural elicitor compounds in the dried powders which could have activated the inactive natural defense system of the treated tomato plants, resulting in lower disease severity as compared to that of control plants. Although we were unable to do so, one way to determine the induction of systemic resistance would be to measure the levels of defense-related enzymes in treated and untreated plants. Defense response-activating elicitors could be found in natural products (Walters *et al*., 2005). At the cellular level, the activated plant defense responses include an oxidative burst leading to cell death, changes in the cell wall composition, synthesis of anti-microbial compounds such as phytoalexins, activation of defense genes and priming of host cells (Kuć, 2006). A coffee-leaf extract formulation induced defense genes in tomato against the bacterial pathogen *Xanthomonas vesicatoria* (Medeiros *et al*., 2009). Cucumber leaves were found to have accumulated antifungal defense compounds against *Pythium ultimum* after their treatment leaf extracts from *Reynoutria sachalinensis* (Daayf *et al*., 2000). Crude water extracts of *Melia azedarach* elicited defense responses from cucumber against *Meloidogyne incognita* and decreased the activities of catalase and peroxidase involved in host H_2O_2 detoxification. Low activities of these enzymes inside the host imply the enhanced defense system of the plant (Cavoski *et al*., 2012). Using aqueous plant extracts of *Hibiscus sabdariffa*, *Punica granatum* and *Eucalyptus globulus*, Hassan *et al*. (2009) demonstrated the elicitation of systemic resistance in potato against BW.

The *in-vivo* results of the higher doses (40 g/kg soil) of the dried powders of *C. procera, A. vasica* and *T. patula* are interesting. These plants proved to be about 60% as effective as the antibiotic in terms of reducing the disease severity and as effective as the antibiotic in terms of enhancing yield and plant growth characters. This implies that the use of the dried powders of these plant species has the potential to become an effective component of the integrated disease management against BW of tomato. Unlike the requirement of expensive drip irrigation system to apply aqueous plant

extracts (Ji *et al*., 2007), dried powders of these medicinal plant species could be directly applied to the root-zones of tomato plants rather than broadcasting them in the whole field. This practice of selective rhizosphere application, which is easily doable in small-scale agricultural systems like those found in Pakistan, will greatly reduce the input costs. Besides being effective against *R. solanacearum* and possibly other plant pathogenic bacteria, these plant species are evergreen, easily available in large amounts and free of cost, which makes them an attractive potential component of integrated disease management against BW and possibly other plant pathogens. These plant species could be used as organic amendments, in the form of green manures or dried powders, to control soil-borne pathogens such as *R. solanacearum* or in the form of aqueous extracts sprays to control foliar bacterial pathogens. Moreover, disease-free tomato transplants could be produced by incorporating dried powders or green manures of medicinal plant species in seed-bed soils. To produce disease-free rice seedlings, neem (*Azadirachta indica*) leaves are routinely incorporated by farmers into rice seed-beds in rice-growing areas of Pakistan. Although the preparation and use of aqueous extracts of medicinal extracts is very convenient and easily affordable by resource-poor farmers of developing countries like Pakistan, many anti-microbial secondary metabolites of plants are more efficiently extracted using organic solvents than water (Al-Obaidi, 2014). Further research is needed to purify and quantify different anti-microbial compounds present in these medicinal plants and to formulate them into commercially usable forms. Research is underway regarding the evaluation of different extraction procedures using organic solvents to identify natural chemical compounds with anti-microbial activity, elucidation of chemical structures in case of discovery of new compounds and testing of these natural compounds against other plant pathogens including bacterial pathogens.

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