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Inhibitory effects of some invasive alien species leaf extracts against tomato (*Lycopersicon esculentum* Mill.) bacterial wilt (*Ralstonia solanacearum*)

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The inhibitory activity of selected invasive alien species leaf extracts against tomato bacterial wilt was studied *in vitro* and greenhouse. *In vitro* inhibitory activity was undertaken in a disc diffusion sensitivity test. Furthermore, aqueous extracts of *Eichhorina crassipes*, *Mimosa diplotricha* and *Lantana camara* and methanolic extract of *Prosopis juliflora* which showed better inhibitory effect *in vitro* were evaluated against *R. solanacearum* in greenhouse on tomato. The plant extracts were applied and evaluated at three different times of application (at the time of inoculation; two days before pathogen inoculation; and two days after pathogen inoculation). The result showed that most of the treatment combinations significantly reduce disease incidence and area under disease progress curve and increase biomass of tomato plants, but the effectiveness of tested plant species depends on the type of plant species and application time. The application of plant extracts at the time of pathogen inoculation resulted in highest reduction of disease development on tomato plants. Aqueous extract of *E. crassipes* was found to be the most effective plant extract in disease suppression and increase above-ground biomass compared to inoculated control. The study revealed that the tested plant species have a potential of inhibiting the development of bacterial wilt on tomato.

Keywords: plant extract; *Eichhorina crassipes*; inhibitory effect; *Mimosa diplotricha*; *Lantana camara*; *Prosopis juliflora*

Introduction

Tomato (*Lycopersicon esculentum* Mill.) is one of the most widely grown vegetables in the world including Ethiopia. In Ethiopia, it is an important cash crop widely cultivated both under irrigation and rain fed conditions (Dandena et al. 2010). But, its production and quality are low which is attributed to different factors.

Diseases caused by different fungal and bacterial pathogens (Getachew et al. 2011), increasing temperature, viral diseases and salinity (Dandena et al. 2010) are among the major limiting factors in sustaining and increasing of tomato production. Bacterial wilt caused by *Ralstonia solanacearum* (Smith) is one of the most destructive plant diseases, which is mainly distributed and damaging in humid climates in the tropical, subtropical and warm temperate regions of the globe (Kurabachew et al. 2007; Mwangi et al. 2008).

In Ethiopia, bacterial wilt was first recorded in 1956 on potato and eggplant around Jimma in the western part of the country (Stewart 1956). Since then, the intensity of the

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disease is increasing from time to time and percentage incidence as high as 45% (Yaynu & Korobko 1986) and 63% (Bekele 1996) in potato and 55% (EARO 2002) in tomato was recorded in major potato-producing parts of the country. Other workers also reported the disease on potato and tomato from Ziway, Ambo, Bako and Guder areas at higher incidences (Tessera et al. 2009).

So far, different control strategies have been employed and suggested such as use of resistant variety, crop rotation, selection of disease free planting material, disinfection of plant materials (Guo et al. 2004), microbial antagonists (Fikre & Zeller 2007), organic soil amendments (Gezahegn et al. 2010; Getachew et al. 2011) and other cultural practices as single or integrated disease management. However, to date there is no single means that would totally manage the disease and fully protect host plants against the pathogen. As a result, the disease is still devastating and causing a significant yield loss to tomato and potato production (Gezahegn et al. 2010). There is, therefore, a need to search alternative management options.

Green plants produce antimicrobial agents by secondary metabolism and can provide valuable sources of natural pesticides; considered as an alternative to synthetic pesticides in organic and sustainable agriculture (Macdonald 2008; Amsalu et al. 2011). Thus, they have been intensively screened and used in different fields of study Rhouma et al. (2009).

Invasive alien species (IAS) is of a great concern in Ethiopia, posing particular problems on a good number of biodiversity sites of the country. The reason to choose and work with IAS is that: (1) IAS may be successful competitors due to resistance towards different pathogens; these plant species may therefore contain active principles to resist plant pathogen attack, (2) extracts of plants has excellent antimicrobial activity, but the cost of cultivating the plant have encouraged the investigation of readily available material such as IAS, and (3) the IAS are used as raw material for plant derived chemicals then there are large quantities of material readily available for use. Moreover, collection of these IAS may protect indigenous plants and, at the same time, may create economic uses and jobs based on these unwanted species. Therefore, the objective of this study was to evaluate the inhibitory activity of selected IAS extracts and their time of application on bacterial wilt disease development on tomato.

Materials and methods

Study area

The research was conducted at Jimma University College of Agriculture and Veterinary Medicine, Jimma, Ethiopia, under laboratory and greenhouse conditions from February to May, 2012.

Collection of plant materials

Fresh leaf samples of the five major IAS, viz. *Parthenium hysterophorus* (Family, Asteraceae); *Lantana camara* (Family, Verbenaceae); *Eichhornia crassipes* (Family, Pontederiaceae); *Mimosa diplotricha* and *Prosopis juliflora*, belonging to the same family, Fabaceae, were collected in July 2011. *P. hysterophorus*, *L. camara* and *M. diplotricha* were collected from natural habitats around Jimma, Ethiopia in August 2011, while *E. crassipes* and *P. juliflora* were collected from natural habitats around Ziway and Afar, Ethiopia, in July 2011, respectively. Collected leaves of each IAS were washed in tap water and surface-sterilised with a 5% sodium hypochlorite solution for

two minutes followed by rinsing thoroughly with sterile water. Then, the plant samples were cut into smaller size, air-dried at room temperature, ground with the help of sterile pestle and mortar into fine powder and kept in refrigerator until use.

Extraction procedure

Crude plant leaf extract was obtained by following standard procedures described by Nduagu et al. (2008). A 50 g of each plant material was separately infused in 250 ml sterilised water, acetone (70%) and methanol (70%), separately, to give 20% (w/v) in a 1000 ml conical flask, kept on shaker for 24 h at 121 rpm. The infusion was filtered afterwards through double-layer cheesecloth followed by Whatman No. 1 filter paper and the organic solvents were evaporated in a hot air oven at 40 °C. After solvent evaporation, the remaining crude extracts were diluted with sterilised water, and kept in air-tight bottle and was put in refrigerator for subsequent use.

Bacterial test organism

Bacterial pathogen was originally isolated from infected tomato plants which were collected from Jimma, Ethiopia, following standard procedures described by Abo-Elyousr and Asran (2009). The single colony technique was adopted to obtain pure cultures and identified as *R. solanacearum* based on their morphological and cultural characteristics on Casamino acids Peptone Glucose (CPG) agar medium as stated in Klement et al. (1990), tomato pathogenicity bioassay and some of the biochemical characteristics. Development of typical wilting symptom was recorded weekly and re-isolation of the bacteria was made on CPG agar medium to confirm the bacteria (Koch's rule). The isolated bacterial culture (strain RsJUCAVMt) was proven to be pathogenic, causing wilt symptoms to tomato plants, catalase positive, and utilised carbohydrate sugars. But, it failed to oxidise sugar alcohols (soribitol and mannitol) suggesting that it belongs to Biovar 2 Race 3 (Hayward 1964; EPPO 2004). The strain was routinely cultured on CPG agar (Smith et al. 1995) at 28 °C for 48 h and temporarily stored in sterile water at room temperature to use for further experiment.

In vitro *antibacterial test*

Antibacterial activity of each plant material extracts was determined following the method described by Abo-Elyousr and Asran (2009). Sterile Petri dishes that are 9 cm in diameter were filled to a depth around 4 mm with CPG agar medium. Thereafter, 100 µl of the bacterial suspension from 24 h old culture was evenly spread over the medium using a sterilised cotton swab as described by Wagura et al. (2011). The inoculated plates were allowed to dry for approximately 5 min and the antibacterial discs (four in number) were placed per plate with a needle. The plates were then incubated at 28 °C for 48 h. After incubation, diameters of inhibition zone around each disc were measured to the nearest millimetre (mm).Three replicates were used for each treatment and treatments were arranged in completely randomised design.

Greenhouse experiment

Based on the efficiency of previous *in vitro* antibacterial result, four plant extracts (aqueous extracts of *E. crassipes, M. diplotericha* and *L. camara* and methanol extract of *P. juliflora*), that scored inhibition zone of >10 mm, were chosen for this experiment.

Tomato seeds

Healthy seeds of tomato plants (*L. esculentum* Mill.) (cv. Chali) which is susceptible to *R. solanacearum* was kindly provided by Melkassa Agricultural Research Centre, Melkassa, Ethiopia. Tomato seeds were surface-sterilised with 2% sodium hypochlorite for 2 min (Guo et al. 2004), and was washed thoroughly with sterilised distilled water and planted in plastic tray filled with the sterilised potting medium.

Inoculum preparation and inoculation

For inoculation, the bacterium was grown on a CPG agar (Smith et al. 1995) at 28 °C for 48 h and wild-type bacterial colonies were harvested and suspended in CPG liquid culture, placed on an orbital shaker at 120 rpm and grown for three days at room temperature. Cultures grown in CPG liquid medium were collected by centrifugation at 6000 rpm for 20 min and washed twice in sterilised water, and serial dilutions were made to adjust bacterial populations approximately to 10^9 CFU^{-1} ml (Yao & Allen 2006).

Soil sterilisation and transplanting

The soil was sterilised in an oven at 82 °C for 30 min by following standard procedures used earlier (How to sterilise potting 2012). Sterilised soil (clay and sand mixture at 1:2 v/v) were filled into the plastic pots having a diameter of 20 cm, which were surface-sterilised with alcohol into which four tomato seedlings were planted.

Four-week tomato seedlings raised in plastic tray were transplanted into pots having sterilised medium. After a week, the potted plants were arranged in three groups in a greenhouse. The plants in the first group were inoculated with the pathogens 10^9 CFU ml^{-1} (30 ml) (Abo-Elyousr & Asran 2009; Sangoyomi et al. 2011) before two days application with plant extract (40 ml of each extract) (Abo-Elyousr & Asran 2009). Plants in the second group were inoculated with the pathogen after two-day application of plant extracts. Plants in the third group were inoculated with the pathogen at the same time of application as the plant extracts. Inoculated and non-inoculated control pots and plants were treated with an equal volume of distilled water. Three replicates were used for each treatment and treatments were arranged in a randomised complete block design.

Disease assessment

Wilt incidence (%), AUDPC (%-days), fresh and dry weight (g), plant height (cm) and *R. solanacearum* population in the rhizosphere soil (CFU/g dry soil) were recorded and calculated. Disease incidence was assessed at weekly intervals for development of bacterial wilt symptoms and calculated as percentage of wilted plants within each treatment according to the formula:

$$\mathrm{WI} = \frac{\mathrm{NPSWS}}{\mathrm{NPPT}} \times 100$$

where WI = wilt incidence, NPSWS = number of plants showing wilt symptoms and NPPT = number of plants per treatment.

Additionally, the area under disease progress curve (AUDPC) was calculated from the percentage of disease incidence and severity according to the midpoint rule (Garrett & Mundt 2000) as:

$$\text{AUDPC} = \sum_{i=0}^{n-1} [0.5(x_i + x_{i+1})][t_{i+1} - t_i]$$

where x_i = incidence at the i^{th} assessment or percentage disease severity, t_i = the time of the i^{th} assessment in days from the first assessment date and n = the total number of days that disease will be assessed. Since incidence (x) was expressed in percent and time (t) in days, AUDPC was expressed in %-days (Campbell & Madden 1990).

Moreover, reduction of wilt incidence and AUDPC was calculated using "PR = [(PC - PT)/PC] × 100"; where PR is percent reduction, PC is percentage value of the control and PT is percentage value of the treatment group. Plant height was recorded by measuring the above-ground part of the plant after two months of pathogen inoculation. Fresh weight was measured by weighing the above-ground cuts of the plant at two-month after inoculation. Dry matter of plants was measured by weighing the oven-dried (at 60 °C for 72 h) above-ground cuts of the plants. Finally, biomass increase was calculated by using the following formula adopted from Seleim et al. (2011).

$$\text{Biomass increase} = \frac{\text{average weight of plants with botanicals} - \text{average weights of control plants}}{\text{average weights of control plants}} \times 100$$

Quantification of R. solanacearum *population in rhizosphere soil*

To assess the effect of selected IAS extracts on the population density of *R. solanacearum* in rhizosphere soil, the procedures described by Fikre and Zeller (2007) were followed. Ten gram of pathogen plant extract-infested soil samples were taken from each pot of treatments two months after pathogen inoculation giving 30 g of soil per treatment. The culturable population of the pathogen was determined by the dilution plate method. The soil was mixed thoroughly, and then 10 g was added to sterile water (1:9, w/v) and shaken for 30 min on a rotary shaker, serial dilutions were made, and 100 µl (0.1 ml) aliquots were spread on the surface of a semi-selective SMSA medium (Englerbrecht 1994). Polymyxin B sulphate (1%), 1% crystal violet, 1% tetrazolium salt, 1% bacitracin, 0.1% penicillin (Sigma), 1% chloramphenol and 1% cycloheximide were used as antibiotics in SMSA medium. After incubating the palates at 28 °C for three days, colonies of *R. solanacearum* were counted and CFU were calculated per gramme (dry weight) of soil.

Data analysis

The experimental data was analysed using the analysis of variance (ANOVA). Statistical analysis was carried out using SAS software version 9.2 (SAS Institute Inc. 2008). Single and interaction effects of factors were determined using the GLM procedure of SAS. Whenever significant interactions were observed between factors, comparisons of means at 5% level were made by Tukey's test. Angular transformation for incidence data and Log transformation (Log_{10} (CFU + 1) for populations of bacteria in the rhizosphere soil was performed before the analysis to normalise variance.

Results

In vitro *antibacterial test*

In vitro antibacterial tests of aqueous, acetone, and methanol leaf extracts of five IAS against the growth of *R. solanacearum* gave significantly different inhibition zone (Figure 1). The *in vitro* antibacterial test result confirmed that most of the tested IAS extracts have antibacterial activity which is revealed through clear inhibition zone created around antibacterial discs against *R. solanacearum*.

All aqueous extracts had significantly inhibited the growth of *R. solanacearum in vitro* compared with control, treated with sterile water. The antibacterial activity of aqueous extracts of five IAS extracts ranged from 8.56 to 26 mm. The highest diameter of inhibition zone was recorded from *E. crassipes* (26 mm) that was statistically different from all other treatment combinations and over the infected control.

Antibacterial assay in greenhouse

Effect on disease incidence

According to the present study, plant extracts, time of application and the interaction of the two had significantly ($p < 0.05$) affected bacterial disease development in terms of disease incidence. Though the applications of all the four plant extracts resulted in reductions of wilt incidence, *E. crassipes* leaf extract was found better in restricting the symptom development of the disease than other three tested plants extracts. Accordingly, simultaneous application of aqueous extract of *E. crassipes* with pathogen inoculation resulted in 75% disease incidence reduction, compared to infected control (Table 1). Other treatments also resulted in a significant reduction of mean wilt incidence in tomato plants compared to none treated, infected control (Table 1).

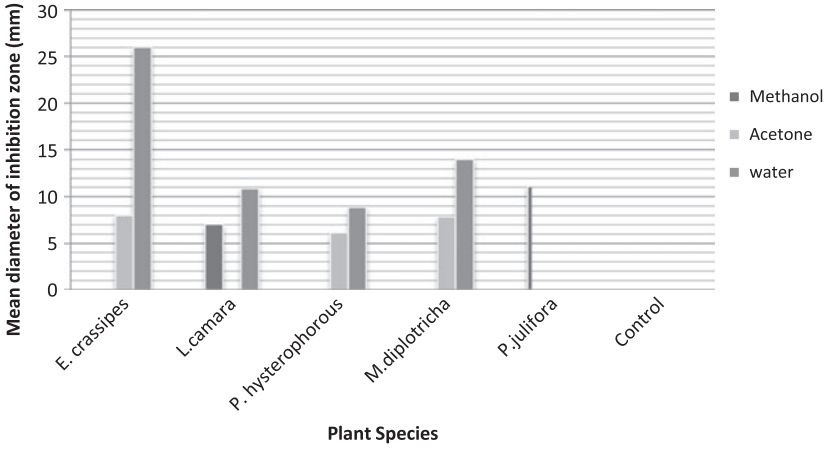

Figure 1. Effect of IAS (*E. crassipes*, *L. camara* and *M. diplotricha* and *P. juliflora*) leaf extracts and type of solvent extractants (methanol, acetone and water) on mean *in vitro* inhibition of *R. solanacearum*.

Effect on AUDPC

A significant interaction between type of botanicals and time of application in terms of incidence area under progress curve (iAUDPC), and severity Area under disease progress curves (sAUDPC) was not evident ($p = 0.2467$ and $p = 0.3053$, respectively). While, main effects of botanicals and time of application had significantly reduced iAUDPC ($p < 0.0001$ and $p = 0.0007$), and sAUDPC ($p < 0.0001$ and $p = 0.0007$), respectively.

Disease severity and wilt incidence expressed as iAUDPC and sAUDPC were significantly reduced by botanicals-treated soil compared to sterile water-treated, infected control (Figure 2). The highest percent reduction of iAUDPC and sAUDPC was exhibited from aqueous extract of *E. crassipes* with 67.52 and 71.35%, respectively, followed by the aqueous extract of *M. diplotricha* with 64.08 and 68.69%, and then *L. camara* aqueous extract with 59.48 and 66.19% (Tables 2 and 3).

Effect on plant height

According to ANOVA, main effects alone and the interaction of the two had significantly increased plant height compared to untreated control. Among the leaf extracts tested, best growth was supported by aqueous extract of *E. crassipes* that scored 53.34, 51.84 and 51.17 cm when it was applied simultaneously, two days after and two days before pathogen inoculation, respectively. The result also indicates that these treatments were statistically similar with the negative control, healthy plants, which scored a plant height of 56 cm (Figure 3). Simultaneous applications of *L. camara* and *M. diplotricha* aqueous extracts were also provided statistically similar result as that of negative control, for which the scores were 50.67 and 53 cm, respectively. The least was recorded from methanol extract of *P. juliflora* leaf extract which scored 42, 42.67 and 42.83 cm when it was applied two days after, two days before and simultaneously with pathogen inoculation though it was statistically different from infected control.

Effect on shoot biomass

The effects of type of botanicals and their time of application on above-ground biomass (fresh and dry weight) per plant are presented in Table 4. Accordingly, the two-way interaction between botanicals and time of application for above-ground fresh weight (AGFW) and above-ground dry weight (AGDW) were significant at 5% probability. Similar to other parameters, aqueous extract of *E. crassipes* applied simultaneously with the pathogen to topsoil gave the highest AGFW with 52.72 g over the inoculated control next to non-inoculated control, healthy plants, and that was statistically differ from other botanicals at $\alpha = 0.05$ (Table 4). Other treatment combinations also yielded a higher AGFW and AGDW over the infected control. But, plants treated with all botanicals at all different time of applications were less in their shoot fresh and dry weights than healthy control plants (Table 4).

Effect on population of R. solanacearum *in the rhizosphere soil*

Two months after inoculation, the availability of bacteria in plant tissue (stem base) was checked by plating bacterial suspensions extracted from 1 cm stem base of symptomless plants on SMSA media. Latent infection was evident in all treatment combinations except in non-infected controls, healthy plants.

Table 1. Interaction effects of selected IAS (*E. crassipes*, *L. camara* and *M. diplotricha* and *P. juliflora*) extracts and their application times, (two days before and after inoculation and simultaneously), on bacterial wilt disease development in tomato caused by *R. solanacearum* under greenhouse condition.

	Mean wilt incidence[y] (%)					
	Application times					
	Before two days		Simultaneous		After two days	
Plant species	DI	PR	DI	PR	DI	PR
E. crassipes	(58.3)50.0^{bc}	41.7	(25.0)30.0^{c}	75.0	(58.3)50.0^{bc}	41.7
L. camara	(66.6)55.0^{b}	33.4	(50.0)45.0^{bc}	50.0	(58.3)50.0^{bc}	41.7
M. diplotricha	(66.6)55.0^{b}	33.4	(33.3)35.0^{bc}	66.7	(58.3)50.0^{bc}	41.7
P. juliflora	(91.6)79.2^{a}	9.4	(66.6)55.0^{b}	33.4	(100.0)88.8^{a}	0.0
Control[z]	(100)88.8^{a}	–	(100)88.8^{a}	–	(100)88.8^{a}	–

PR was calculated based on "PR = [(DIC − DIT)/DIC] × 100"; where PR is percent reduction, DIT is disease incidence by the treatment group and DIC is disease incidence by the control. Percent reductions were calculated from the actual data.

[y]Values in the same column followed by the same letter(s) are not significantly different at ($\alpha = 0.05$) according to Tukey test and values in brackets are actual data before transformation.

[z]Pathogen inoculated and sterile water treated instead of botanicals.

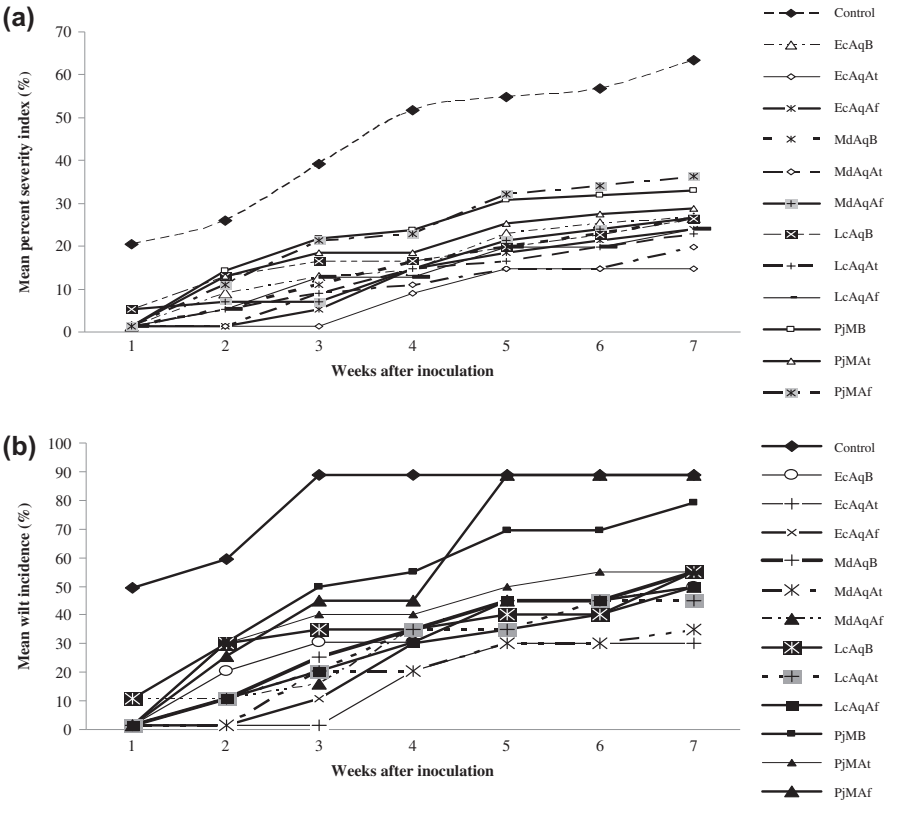

Figure 2. Bacterial wilt development as mean percent severity index (a) and wilt incidence (b) on tomato plants treated with selected plant extracts at different time of application under greenhouse conditions. Control-uninoculated; EcAqB–*E. crassipes* aqueous extract applied 2 days before inoculation; EcAqAt–*E. crassipes* aqueous extract applied at the time of inoculation; EcAqAf–*E. crassipes* aqueous extract applied 2 days after inoculation; MdAqB–*M. diplotricha* aqueous extract applied 2 days before inoculation; MdAqAt-*M. diplotricha* aqueous extract applied at the time of inoculation; MdAqAf-*M. diplotricha* aqueous extract applied 2 days after inoculation; LcAqB–*L. camara* aqueous extract applied 2 days before inoculation; LcAqAt–*L. camara* aqueous extract applied at the time of inoculation; LcAqAf–*L. camara* aqueous extract applied 2 days after inoculation; PjMB–*P. juliflora* methanol extract applied 2 days before inoculation; PjMAt–*P. juliflora* methanol extract applied at the time of inoculation; and PjMAf–*P. juliflora* methanol extract applied 2 days after inoculation.

Table 2. Effect of selected IAS (*E. crassipes*, *L. camara* and *M. diplotricha* and *P. juliflora*) extracts on iAUDPC and sAUDPC under greenhouse condition.

Plant species	Mean iAUDPC[y] (%-days)	PR (%)	Mean sAUDPC[y] (%-days)	PR (%)
E. crassipes	1318.3[c]	67.52	669.37[c]	71.35
L. camara	1645.0[c]	59.48	790.23[c]	66.19
M. diplotricha	1458.3[c]	64.08	731.84[c]	68.69
P. juliflora	2619.2[b]	35.48	1157.41[b]	50.48
Control[z]	4060.0[a]	–	2337.58[a]	–

Percent reduction (PR) was calculated based on "PR = [(AUDPCC − AUDPCT)/AUDPCC] × 100".
[y]Values in the same column followed by the same letter(s) are not significantly different at (α = 0.05) according to Tukey test.
[z]Pathogen inoculated and sterile water treated instead of botanicals.

Table 3. Effects of application time of selected IAS (*E. crassipes*, *L. camara* and *M. diplotricha* and *P. juliflora*) extracts on iAUDPC and sAUDPC under greenhouse condition.

Application time	Mean iAUDPC[z] (%-days)	Mean sAUDPC[z] (%-days)
Before two days	2422.0[a]	1228.96[a]
Simultaneous	1921.5[b]	1017.48[b]
After two days	2317.0[a]	1165.42[a]

[z]Values in the same column followed by the same letter (s) are not significantly different at ($\alpha = 0.05$) according to Tukey test.

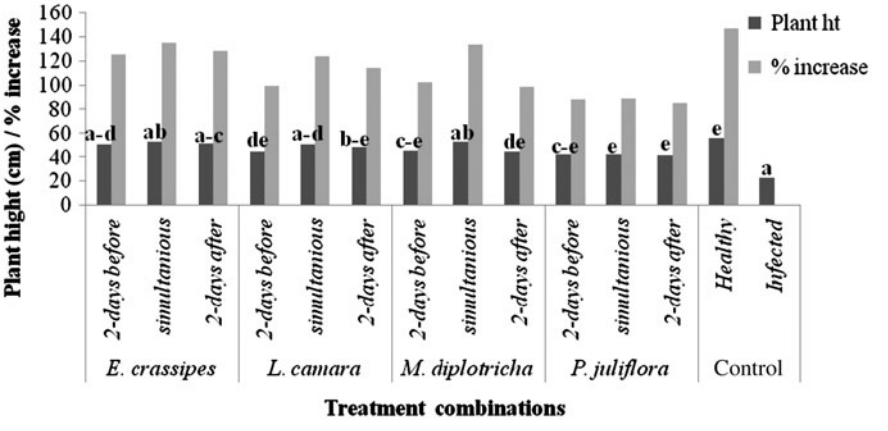

Figure 3. Interaction effect of IAS (*E. crassipes*, *L. camara M. diplotricha* and *P. juliflora*) extracts and their time of applications (two days before and after inoculation and simultaneously) on height of tomato plants infected with *R. solanacearum* under greenhouse condition.

The population densities of *R. solanacearum* in the rhizosphere soil were assessed two months after inoculation. The number of culturable bacteria recovered from rhizosphere soil two months after inoculation was significantly lower (2.1×10^3) in the rhizosphere soil drenched by *E. crassipes* simultaneously treated with pathogen inoculation pot compared to control treatment (3.22×10^7)/g. dry soil. Population densities of *R. solanacearum* [$\log_{10}$ (CFU + 1)/g, dry soil] in the rhizosphere soil after incorporation of botanicals are shown in Table 5. Crude aqueous extract of *E. crassipes* gave the highest percent reduction (50%) in *R. solanacearum* population, followed by *L. camara* (47.72%) and then *M. diplotricha* (44.69%) when applied simultaneously with pathogen inoculation.

Discussion

Synthetic pesticides are nowadays widely used for the control of plant diseases throughout the world because of their higher effectiveness in controlling disease causing organisms. However, excessive and unsystematic application of these chemicals has created several environmental and health hazards and some phytopathogens have developed resistance (Rhouma et al. 2009). Plants produce antimicrobial agents by secondary metabolism to protect themselves from pathogen attack, and therefore many plant species possess substantial antimicrobial activity (Macdonald 2008).

Table 4. Interaction effect of some IAS (*E. crassipes*, *L. camara* and *M. diplotricha* and *P. juliflora*) extracts and their time of application (two days before and after inoculation and simultaneously) on above-ground fresh and dry weight and biomass increase of tomato plants under greenhouse conditions.

	Above-ground biomass[x] (g)											
	Application times											
	Before two days				Simultaneously				After two days			
Plant species	AGFW	BI	AGDW	BI	AGFW	BI	AGDW	BI	AGFW	BI	AGDW	BI
E. crassipes	33.1 ± 1.1^{e}	276	8.9 ± 0.4^{d}	219	52.7 ± 1.4^{b}	499	11.9 ± 0.1^{b}	328	40.0 ± 1.8^{d}	355	9.9 ± 0.2^{c}	256
L. camara	42.8 ± 1.3^{d}	387	8.6 ± 0.3^{de}	207	48.3 ± 0.8^{c}	449	9.3 ± 0.5^{cd}	235	31.3 ± 0.9^{e}	256	9.0 ± 0.5^{d}	222
M. diplotricha	31.3 ± 0.3^{e}	256	7.9 ± 0.2^{e}	184	48.7 ± 1.5^{c}	454	9.2 ± 0.4^{cd}	231	30.5 ± 0.9^{e}	247	8.6 ± 0.6^{de}	208
P. juliflora	16.9 ± 0.4^{g}	92	3.7 ± 0.1^{g}	31	33.1 ± 0.9^{e}	277	6.7 ± 0.2^{f}	139	25.64 ± 1.1^{f}	191	6.2 ± 0.1^{f}	121
Control[y]	8.8 ± 0.2^{h}	–	2.8 ± 0.1^{g}	–	8.8 ± 0.2^{h}	–	2.8 ± 0.1^{g}	–	8.8 ± 0.2^{h}	–	2.8 ± 0.1^{g}	–
Healthy[z]	113.9 ± 1^{a}	1195	15.5 ± 0.3^{a}	454	113.9 ± 1^{a}	1195	15.5 ± 0.3^{a}	454	113.9 ± 1.0^{a}	1195	15.5 ± 0.3^{a}	454

PI was calculated based on "PI = [(AGFWC − AGFWT)/AGFWC] × 100"; where BI is Biomass increase, AGFWT is above-ground fresh weight by the treatment group, AGFWC is above-ground fresh weight by the control and AGDW is above-ground dry weight.

[x]Values followed by the same letter(s) for a parameter are not significantly different at ($\alpha = 0.05$) according to Tukey test.

[y]Pathogen inoculated and sterile water treated instead of botanicals.

[z]Neither pathogen inoculated nor treated with botanicals.

Table 5. Interaction effect of selected IAS (*E. crassipes*, *L. camara M. diplotricha* and *P. juliflora*) extracts and their time of application (two days before and after inoculation and simultaneously) on *R. solanacearum* population in rhizosphere soil and percent reduction on tomato plants under greenhouse condition.

	Log $_{10}$ (CFU + 1)/g.dry soil[y]					
	Application times					
	Before two days		Simultaneously		After two days	
Plant species	CFU	PR	CFU	PR	CFU	PR
E. crassipes	4.50 ± 0.10^{e}	31.82	3.30 ± 0.10^{g}	50.00	4.65 ± 0.05^{de}	29.54
L. camara	4.74 ± 0.05^{cd}	28.18	3.45 ± 0.05^{fg}	47.72	4.86 ± 0.04^{cd}	26.36
M. diplotricha	4.87 ± 0.04^{cd}	26.21	3.65 ± 0.05^{f}	44.69	4.75 ± 0.05^{cd}	28.03
P. juliflora	5.95 ± 0.05^{b}	9.84	4.87 ± 0.06^{c}	26.21	5.96 ± 0.05^{b}	9.69
Control[z]	6.60 ± 0.10^{a}	–	6.60 ± 0.10^{a}	–	6.60 ± 0.10^{a}	–

PR was calculated based on "PR = [(CFUC − CFUT)/CFUC] × 100"; where PR is percent reduction, CFUT is colony-forming units by the treatment group and CFUC is colony-forming unit by the control.
[y]Values in the same column followed by the same letter(s) are not significantly different at (α = 0.05) according to Tukey test.
[z]Pathogen inoculated and sterile water treated instead of botanicals.

The result of the present study showed that among tested IAS crude extracts, aqueous extract of *E. crassipes* consistently restricts the disease development on tomato plants under greenhouse conditions. Accordingly, 75% reduction in wilt incidence of bacterial wilt was recorded on tomato plants treated with leaf extract of *E. crassipes* in the greenhouse experiment when it was applied simultaneously with pathogen inoculation. Moreover, the highest percent reductions of AUDPC and bacteria population in the rhizosphere soil as well as the highest percent increase of AGFW, AGDW and plant height were also exhibited from aqueous extract of *E. crassipes*. The antibacterial activity of *E. crassipes* may be attributed to various chemicals detectable in its extracts. Phytochemical compositions present in crude extracts from *E. crassipes* are tannins, phlobatannin, steroid, terpenoid, alkaloid, flavonoid, phenolic contents, quinone, anthraqninone and cardiac glycosides as reported by (Dubey & Lata 2010; Aboul-Enein et al. 2011). Thus, the crude extract contained different antibacterial substances with variable efficiencies and mode of actions may act synergistically in inhibition of bacterial growth and in restricting bacterial wilt symptom development.

The antibacterial activity of crude aqueous leaf extracts of *L. camara* was also evaluated on tomato plants and resulted in the reduction of wilt incidence, AUDPC and *R. solanacearum* population in the rhizosphere soil, over the infected control. This suggested the presence of active principles in leaves of *L. camara* and could be attributed to its antibacterial activity. According to Mary (2011), phytochemical analysis of the methanol and distilled water extracts revealed that leaves of *L. camara* contained saponin, tannin, steroids and flavonoid and all are reported to possess antibacterial activity. In addition, the leaves are rich in essential oils and phenolic compounds like aesulin, quercetin, isorhamnetin, fisetin, gossypetin, tricine and aesculetin and triterpenoids (Sousa et al. 2010).

Aqueous crude extracts of *M. diplotricha* also consistently reduced mean wilt incidence in tomato plants and *R. solanacearum* population density in the rhizosphere soil in all three time of application being simultaneous application is performed better. This is comparable with Wiersum (1983), who reported that use of *M. diplotricha* as a

cover crop, in tobacco plantations in Sumatra, reduced the incidence of slime disease, caused by *R. solanacearum*, to very low levels. Moreover, the result of the current study revealed that methanol extract of *P. juliflora* was inconsistent in its antibacterial efficacy. Hence, the least reduction of mean wilt incidence and AUDPC were recorded in tomato plants drenched with methanol extract of *P. juliflora,* compared with other treatments in all application time. But, higher biomass increase in terms of plant height, AGFW and AGDW was exhibited by crude methanol extract of *P. juliflora* with varied results at different application time, compared to infected control. The antimicrobial potential of extracts from *P. juliflora* has been evaluated by many researchers against different micro-organisms and supported the finding of this study. As indicated by Ahmad (1991), *P. juliflora* leaf extracts contained alkaloids; juliflorine, juliflocine and benzene insoluble alkaloidal fraction, and all these alkaloids were found to be possessingd significant antibacterial activity.

Barsagade and Wagh (2010) indicated antimicrobial activities of plant and weed extracts may exist in a variety of different components, including aldehyde and phenolic compounds. Naturally occurring combination of these compounds can be synergistic and often result in crude extracts having greater antimicrobial activity than the purified, individual constituents. Cowan (1999) indicated that substrate deprivation, membrane disruption, bind to adhesives, complex with cell wall, enzyme inhibition and inactivation, metal ion complexation and intercalate into cell wall and/or DNA are the possible mechanisms of plant secondary metabolites against different micro-organisms.

According to the present study, application of crude aqueous extracts and methanol extract of selected IAS at different time of application significantly reduced bacterial wilt development and increased biomass on tomato plants, compared to infected control. But, varied results were found based on plant species used and their application time. This variation in restricting disease development between IAS and their application time might be due to the fact difference in chemical compositions of the extracts, membrane permeability of the target pathogen, difference in efficacy and durability of extracts in the soil. This was supported by the work of Hassan et al. (2009), who reported that soil drenching of some aqueous plant extracts variably and significantly reduced the disease severity of bacterial wilt, caused by *R. solanacearum*, on potato plants compared with inoculated control under both greenhouse and field conditions. In addition, as indicated by Pradhanang et al. (2003), thymol, palmarosa and lemongrass oil as soil fumigants were suppressed bacterial wilt incidence of tomato caused by *R. solanacearum* in greenhouse pot experiments.

The result of the study also revealed that none of tested botanicals increased plant height significantly compared with uninoculated, healthy plants. Moreover, all botanically treated plants scored lesser AGFW and AGDW compared to uninoculated control, healthy plants. This ascribed that botanicals by themselves do not increase biomass (promote plant growth) rather they decrease the negative impact of the pathogen, stunting nature of the disease, on plant growth, which also affects fresh weight and dry weight of plants. However, significantly higher biomass (plant height, AGFW and AGDW) was recorded from plants treated with botanicals compared with untreated plants. This is in accordance with Opara and Wokocha (2008), who reported that some aqueous plant extracts increased plant growth parameters including plant height at varying degrees over the infected control against *Xanthomonas campesrtis* pv. *vesicatoria* under field experiment. The results of the present study are in agreement with those reported by Abo-Elyousr and Asran (2009), who reported that treated tomato plant with different plant extracts at different application time exhibited lesser shoot fresh and dry

weight than healthy plants, uninoculated control. Our results are in disagreement with those reported by El-Ariqi et al. (2005), who reported that treated potato plant with different plant extracts provided higher shoot and root fresh weight, compared with uninoculated control.

The number of culturable bacteria recovered from rhizosphere soil two months after inoculation was significantly lower in the rhizosphere soil drenched with botanicals. Varied percent reduction of *R. solanacearum* population in the rhizosphere soil was recorded from soil drenched with aqueous extract of *E. crassipes, L. camara*, *M. diplotricha* and methanolic extract of *P. juliflora* after two months of pathogen inoculation. The differences may be due to the tested botanicals variation in their bacteriostatic and bactericidal effects on the cells of *R. solanacearum* which in turn affect disease development on tomato plants. This is in line with Fikre and Zeller (2007), who reported that disease development in plants was closely related to population dynamics of *R. solanacearum* in the soil. Similar results were found by Pradhanang et al. (2003) and Paret et al. (2010), who reported that soil drenching with thymol, palmarosa and lemongrass oil suppressed *R. solanacearum* populations in soil and reduced bacterial wilt incidence at varying degrees in greenhouse pot experiments. Investigations on the mechanisms of disease suppression by plant products have suggested that the active principles present in plant extracts may either act on the pathogen directly or induce systemic resistance in host plants resulting in a reduction of disease development (Hassan et al. 2009).

Overall, the results indicated that drenching topsoil with aqueous crude extracts of *E. crassipes*, *M. diplotricha*, *L. camara* and methanolic extract of *P. juliflora* at different application time have the potential to suppress the bacterial wilt incidence and severity, and *R. solanacearum* populations in soil. Similarly, drenching of botanicals in the soil provided biomass (fresh weight, dry weight and plant height) increases over the non-treated control, with simultaneous application time being the most effective. It is interesting to notice that the compounds in crude extracts of IAS showed antibacterial activity, which gives new opportunities to improve control strategies, against tomato bacterial wilt diseases, in organic and sustainable agriculture. However, a better understanding of their mechanisms of action could contribute to an optimal utilisation alone or in the development of integrated disease management approach against bacterial wilt of tomato. Further research is necessary to determine the identity of the antibacterial compounds of these IAS extract active compounds. The effectiveness and practical application of these effective plant extracts also need to be studied further under open field conditions.

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