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Effect of botanicals on *Ralstonia solanacearum* and bacterial wilt incidence in tomato

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Abstract
Tomato is considered as the second most important vegetable crop in India and major constraint in its production is bacterial wilt caused by *Ralstonia solanacearum*. The management of the disease is difficult and the chemicals are least effective and are hazardous. Hence, an alternate method of control needs to be developed which is effective, cheap and eco friendly. The water extract from only two botanicals *viz*., *Ocimum gratissimum* and *Tylophora asthmatica* were effective in inhibiting the growth of *Ralstonia solanacearum. O. gratissimum* extract showed highest inhibition zone of 28.66 mm at 1:0 dilution and had inhibitory effect upto 1:10 dilution with 22.66 mm inhibition zone. Alcohol extract of *O. gratissimum* was the most effective in inhibiting the growth of *R. solanacearum* followed by *C. gigentea*, *O. sanctum*, *T. asthmatica*, *N. sativa* and *R. graveolens*. Alcohol extract *Ocimum gratissimum* was found more efficacious than *O. sanctum* in delaying the onset of wilt disease wherein 100 per cent wilt incidence was observed at 56 DAI.

Keywords: Medicinal plants*: Ralstonia solanacearum:* tomato: Inhibition zone

Introduction
Tomato (*Solanum lycopersicum* L.) belongs to the family *Solanaceae* and is considered as the second most important vegetable crop next to potato. Tomato has an excellent nutritional profile owing largely to its balanced mixture of minerals (Potassium, Calcium, Phosphorus, Iron and Zinc), vitamins (A, B1, B2, B6, C, E and K), antioxidants such as carotenoids and poly phenolic compounds and carbohydrates (Khoso, 1994) [7].

The major restraint to tomato production in India is bacterial wilt caused by *R. solanacearum* (Yabuuchi *et al*., 1995). [16] *R. solanacearum* also causes diseases in other economically important crops such as potato, eggplant, chilly and non *solanaceous* crops such as banana and groundnut (Anuratha *et al.,* 1990) [2] and thus noted to be a major constraint in the production of many important vegetables, fruit, and cash crops. The yield loss may vary between 10.8 and 90.6 percent depending on the environmental conditions and the stage at which infection occurs (Kishun, 1987). [8] Bacterial Wilt poses a constant threat to tomato in Karnataka, Madhya Pradesh, Maharashtra and West Bengal in India. The pathogen infects susceptible plants in roots, usually through wounds (Pradhanang *et al.*, 2005) [12] and colonizes within the xylem preventing the water movement into upper portion of the plant tissue (Kelman, 1998). [6] Bacterial wilt is among the most difficult diseases to control. Although crop rotation with non host crops may suppress soil borne populations of the pathogen (Ahmed *et al.,* 2000), [1] the pathogen survive in the soil in association with weed hosts, which impairs the effect of crop rotation.

The use of chemicals has not been effective in the control of bacterial wilt of tomato because the pathogen is a soil borne and is systemic in its nature. The use of copper based bactericides and antibiotics seldom gave satisfactory control. Botanicals because of their natural origin are biodegradable and they do not leave toxic residues or by-products to accumulate in the environment. Therefore under this scenario, botanical pesticides seem to be ideal candidate to be exploited in management of bacterial wilt of tomato in view of the safety, renewable nature, cost effective and high target specificity. Hence an investigation was conducted to study the effect of extracts from few important medicinal plants against *R. solanacearum*.

Material and Methods

Selection of medicinal plants

Medicinal plants mentioned in table 1 which were reported to contain some antibacterial constituents and being used in Indian system of medicine (Kamala Ramachandran *et al*., 1986) [5] were selected to screen for their antibacterial properties against *R. solanacearum* causing bacterial wilt of tomato. These plants were collected from College of Agriculture, Mandya.

Isolation of Pathogen

Tomato plants showing typical symptoms of vascular discolouration caused by *R. solanacearum* were collected. The presence of the pathogen in the host was confirmed by ooze test. The bacterium was isolated on solidified triphenyl tetrazolium chloride (TZC) agar medium. The tissue from the lower part of the infected stem were cut into small pieces aseptically, and surface sterilized in 70 percent alcohol and were washed in three series of sterile water to remove traces of alcohol. The infected tissue pieces were then suspended in a test tube containing sterilized water for 10 minutes. The bacterial suspension was spread on the surface of TZC medium with spreader. The inoculated plates were incubated at 30^0C for 48 hours. The plates were observed for the development of well-separated virulent colonies. It was purified by picking the highly virulent colonies and streaked on the surface of TZC medium contained in Petri dishes. Three to four loopful of well-separated virulent colonies were suspended in sterile distilled water taken in vials. The vials were stored at 5^0C, and served as stock culture for further studies.

The bacterium isolated from diseased plant was identified on the basis of morphological, cultural and biochemical characteristics prescribed by Bradbury (1986) [3] and Schaad and Stall (1998) [14].

Method of Extraction

Two most common methods used in extraction were followed to extract the antimicrobial components contained in eight different plant species in order to screen for their antimicrobial property against tomato bacterial wilt pathogen were (1) Water extract method (2) Alcohol extract method.

Protocol for water extract

The economic parts of the plants noted in table 1 were used for the purpose of extraction. 50g of leaves or seed as the case may be were taken and cut into small pieces under aseptic condition. The sample was put into waring blender containing 50ml sterilized distilled water at a ratio 1:1 (water: plant material). The sample was spun at low speed for 10-15 minutes in a coffee warring blender till the material formed to fine texture. The blended material was then squeezed through a sterilized muslin cloth so as to get a crude liquid extract. The crude extract was filtered through Whatman no 1 filter paper followed by sterilized Seitz filter. The sterilized filterate was collected in sterilized glass tubes and the tubes were sealed under aseptic condition and labelled as "WE". The water extract was kept at 5ºC in a refrigerator for further use.

Protocol for alcohol extract

Fifty gram of the economic parts of the respective plant was mixed with a small quantity of 70 per cent ethyl alcohol and macerated in a pestle and morter under aseptic condition. The material was blend to fine texture, transferred to a beaker and the final volume was made up to 50ml with 70 per cent ethyl alcohol in the ration of 1:1 (plant material: alcohol). The beaker was kept overnight under refrigerated condition. Alcohol extract was squeezed through muslin cloth, then passed and finally sterilized through Seitz filter apparatus. The sterilized filterate was collected in sterilized glass tubes and the tubes were sealed under aseptic condition and labelled as "AE". The alcoholic extract was stored at 5ºC in a refrigerator for further use.

In vitro evaluation of plant extracts

Both water and alcohol extracts of the medicinal plants were screened at different dilutions viz., 1:0 (undiluted), 1:1, 1:10, 1:100, 1:1000. The efficacy of the extracts were tested by the zone of inhibition assay technique against *Ralstonia solanacearum* causing bacterial wilt of tomato. A heavy suspension of the test bacteria ($7x10^8$cfu/ml) was seeded to the sterilized nutrient agar medium by mixing the bacterial cultural with the cooled nutrient agar (45-50ºc) in a 500ml Erylenmeyer flask. The seeded medium was poured on sterilized Petri plates and allowed to solidify.

Sterilized filter paper disc (Whatman no.1) measuring 10mm diameter were soaked for 10 minutes in undiluted (1:10) and diluted (1:1, 1:10, 1:100 and 1:1000) plant extracts and placed on the surface of seeded nutrient agar medium contained in the Petri plates in marked position. The inoculated plates were incubated first at 4ºC for 4 hours so as to allow the diffusion of the extract into the medium. The plates were then transferred to incubator maintained at 30ºC and incubated for 48 hours. Observations were recorded on the zone of inhibition produced around the filter paper disc in each plant extract at different dilutions, by measuring the diameter of the inhibition zone.

Effect of plant extracts on wilt of tomato caused by *R. solanacearum*

From the stock culture of the virulent colonies, a loopful of bacterial culture was taken and streaked on the surface of TZC medium contained in Petri dishes. The highly virulent colonies were selected and a loopful of the inoculums was added to the sterilized nutrient broth taken in the Erlenmeyer flask for the purpose of multiplication of the virulent culture and the inoculated flasks were incubated for 48 hours at 30ºC. The concentration of the cells in the suspension was adjusted to $7x10^8$ cfu/ml turbidometrically.

Tomato (cv. Pusa Ruby) seedlings were raised in the nursery by sowing tomato seeds in sterilized soil contained in plastic trays. Twenty five days old seedlings were inoculated with the bacterial suspension by root injury inoculation technique. Then the inoculated tomato seedlings were transplanted to 6" pots containing sterilized soils.

The inoculated seedlings were drenched a day after transplanting with alcohol extract (1: 10 dilution) obtained from *Ocimum gratissumum* and *O. sanctum* which were found to be effective in inhibiting the growth of *R. solanacearum* in-vitro, along with sterile water and streptocycline (400ppm). The observations were recorded on percentage wilt incidence at weekly intervals.

Results and Discussion

Effect of water extract of medicinal plants against *R. solanacearum*

Water extracts of *O. gratissimum* and *T. asthmatica* had inhibitory effect against *R. solanacearum* and rest of plant extracts tested had no effect at all. *O. gratissimum* extract showed highest inhibition zone of 28.66 mm at 1:0 dilution

and had inhibitory effect upto 1:10 dilution (22.66 mm) which was far better than the control (14.33 mm). Whereas, *T. asthmatica* was effective only upto 1:1 dilution and was superior to control at 1:0 dilution with inhibition of 17.33 mm (table 2). Saha *et al*. (2013) [13] reported inhibitory effect of extracts of five *Ocimum* species against Gram-positive and Gram-negative bacteria and few plant pathogenic fungi.

Effect of alcohol extract of medicinal plants against *R. solanacearum*

The alcohol extracts of six medicinal plants viz., *O. gratissimum*, *O. sanctum*, *T. asthmatica*, *R. graveolens*, *C. gigentea*, and *N. sativa* exhibited inhibitory effect whereas, *S. persica* and *T. cardifolia* had no such effect (table 3). Among the botanicals tested, *O. gratissimum* was the most effective in inhibiting the growth of *R. solanacearum* followed by *C. gigentea*, *O. sanctum*, *T. asthmatica*, *N. sativa* and *R. graveolens*. Extract of *O. gratissimum* was effective up to 1:100 dilution and produced inhibition zones of 29.66, 28.33, and 19.33 mm at 1:0, 1:1 and 1:100 dilution respectively which was superior when compared streptocycline (14.33 mm) which served as control. *O. sanctum* and *T. asthmatica* extracts were inhibitory up to 1:10 dilutions whereas, *R. graveolens*, *C. gigentea* and *N. sativa* extracts were effective upto 1:1 dilutions. But *S. persica* and *T. cardifolia* extracts showed no inhibitory effect at all the dilutions tested against the pathogen. Murthy *et al* (2014) [9] observed solvent extracts of *Ocimum sanctum* inhibited the growth of *R. solanacearum*. Similarly, Ponnanikajamideen *et al*., (2013) [11] found that *T. asthmatica* extracts obtained from different extracts showed inhibitory effect against different strains of bacteria. The alcohol and water extracts of *R. graveolens* exhibited inhibitory activity against many Gram negative bacterial and plant pathogenic fungi tested (Pandey *et al*., 2011). [10] The strong antibacterial activity of essential oil of *N. sativa* seeds was demonstrated against both Gram-positive and Gram-negative bacteria and maximum inhibitory activity was recorded against *Bacillus subtilis* (El-Kamali *et al*., 1998). [4] Vijai Pal *et al*. (1993) [15] also observed inhibitory activity against three *Erwinia* spp. causing soft rot of potato by the extract of *Calotropis procera*, thus confirmed the result obtained during present investigation.

Effect of botanicals on wilt disease incidence

Plant extracts showing very good antimicrobial activity in in-vitro assay were selected and tested to study the effect of the development of bacterial wilt of tomato caused by *R. solanacearum* under *in –vivo* condition. Alcohol extracts (1:10) protected the plants initially from wilt disease upto a period of 20-25 days, however, no protection was obtained at a later stage of disease development as 100 per cent wilt incidence was observed after 40 DAI and 56 DAI in tomato plants treated with *O. sanctum* and *O. gratissimum* respectively. *Ocimum gratissimum* was found more efficacious than *O. sanctum* in delaying the onset of wilt disease i.e., 100 per cent wilt incidence was observed at 56 DAI in *O. gratissimum*, whereas, in *O. sanctum* wilt incidence was observed at 42 DAI. In case of streptocycline (400ppm) only 30 per cent wilt incidence was observed at 56 DAI (table 4). Bora and Semual (1998) who found that the aqueous extracts of *Aloe vera*, *Psidium guajava* and *Allium sativum* applied as root dip treatment to tomato seedlings gave a very good protection to tomato plants from the incidence of wilt and reduced the wilt by 80, 76 and 72 per cent respectively.

Conclusions

The present investigation revealed that extracts of many medicinal plants had inhibitory effect against *R. solanacearum*. Further, alcohol extracts were more effective than water extracts as in alcohol extract, more number of phytochemicals liberated and their efficacy were enhanced against the pathogen. Hence these could be exploited as an alternate management strategy for chemical pesticides in the management of bacterial wilt of tomato and other members of the *Solanaceae* family that are often infected by *R. solanacearum.* The future studies should focus on identification and elucidation of the active principles present in medicinal plants having potential antimicrobial properties.

Table 1: List of medicinal plants used to test their antimicrobial properties against *Ralstonia solanacearum*

S. No	Common Name	Scientific Name	Family	Part used for extraction
1	Antamul	*Tylophora asthmatica W. & A.*	Asclepiadaceae	Leaves
2	Mudar	*Calotropis gigentea L*	Asclepiadaceae	Shoot
3	Holy basil	*Ocimum sanctum L*	Lamiaceae	Leaves
4	Clocimum	*Ocimum gratissimum L*	Lamiaceae	Leaves
5	Tinospora	*Tinospora cardifolia Willd.*	Menispermaceae	Leaves
6	Black Cumin	*Nigella sativa L.*	Ranunculaceae	Seeds
7	Garden Rue	*Ruta graveolens L.*	Rutaceae	Shoot
8	Meswak	*Salvadora persica L.*	Salvadoraceae	Stem

Table 2: Effect of water extracts of medicinal plants against *Ralstonia solanacearum*

Dilution	Zone of inhibition (mm)							
	O. gratissimum	***O. sanctum***	***T. asthmatica***	***R. graveolens***	***C. gigentea***	***N. sativa***	***S. persica***	***T. cardifolia***
1:0	28.66 (5.45)	0.00 (1.00)	17.33 (4.28)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
1:1	26.66 (5.26)	0.00 (1.00)	13.33 (3.78)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
1:10	22.66 (4.86)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
1:100	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
1:1000	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Streptocycline 400 ppm. (control)	14.33 (3.92)	14.33 (3.92)	14.33 (3.92)	14.33 (3.92)	14.33 (3.92)	14.33 (3.92)	14.33 (3.92)	14.33 (3.92)

(Figures in parenthesis are square root transformed values)

	S.Em+	CD (1%)
Factor A	0.0197	0.0556
Factor B	0.0197	0.0556
A x B	0.0483	0.1362

Table 3: Effect of alcohol extracts of medicinal plants against *Ralstonia solanacearum*

Dilution	Zone of inhibition (mm)							
	O. gratissimum	***O. sanctum***	***T. asthmatica***	***R. graveolens***	***C. gigentea***	***N. sativa***	***S. persica***	***T. cardifolia***
1:0	29.66 (5.54)	23.66 (4.97)	19.33 (4.51)	16.66 (4.20)	24.33 (5.03)	18.00 (4.36)	0.00 (1.00)	0.00 (1.00)
1:1	28.33 (5.42)	20.33 (4.62)	15.66 (4.08)	15.00 (4.00)	21.33 (4.72)	12.66 (3.70)	0.00 (1.00)	0.00 (1.00)
1:10	24.33 (5.03)	17.33 (4.28)	12.33 (3.65)	0.00 (1.00)	0.00 (1.00)	12.00 (3.61)	0.00 (1.00)	0.00 (1.00)
1:100	19.33 (4.51)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
1:1000	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
Streptocycline 400 ppm. (control)	14.33 (3.92)	14.33 (3.92)	14.33 (3.92)	14.33 (3.92)	14.33 (3.92)	14.33 (3.92)	14.33 (3.92)	14.33 (3.92)

(Figures in parenthesis are square root transformed values)

	S. Em+	CD (1%)
Factor A	0.0120	0.0338
Factor B	0.0120	0.0338
A x B	0.0294	0.0828

Table 4: Effect of plant extracts on the incidence of bacterial wilt of tomato caused by *Ralstonia solanacearum*

Percent wilt incidence								
Botanicals (alcoholic extract)	**Days after inoculation (DAI)**							
	7	14	21	28	35	42	49	56
Ocimum gratissum	0	0	0(0.71)	20.(0.91)	20(0.91)	50(1.61)	70(1.34)	100(1.58)
Ocimum sanctum	0	0	0(0.71)	60(1.26)	80(1.44)	100(1.58)	100(1.58)	100(1.58)
Streptocycline (400ppm)	0	0	0(0.71)	0(0.71)	0(0.71)	20.(0.91)	20.(0.91)	30(1.02)
Water (control)	0	0	80(1.44)	90(1.51)	100(1.58)	100(1.58)	100(1.58)	100(1.58)
S. Em+			0.0437	0.1164	0.0789	0.1074	0.1038	0.0634
C. D. @ (5%)			0.1345	0.3588	0.2433	0.3310	0.3200	0.1954

(Figures in parenthesis are square root transformed values)

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