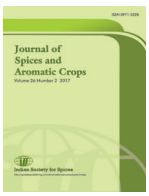
Journal of Spices and Aromatic Crops
Vol. 26 (2) : 95-100 (2017)
doi : 10.25081/josac.2017.v26.i2.879

Indian Society for Spices



Evaluation of the antibacterial activity of mango ginger rhizome extracts against bacterial wilt pathogen *Ralstonia solanacearum*

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Received 15 April 2017; Revised 26 May 2017; Accepted 31 May 2017

Abstract

The Indian mango ginger (*Curcuma amada* Roxb.) is a perennial rhizomatous herb with a raw mango flavour. It is resistant to bacterial wilt disease. In order to understand the disease resistance mechanism of mango ginger the hexane, chloroform and methanol extracts (5, 10, 25, 50 and 100 mg mL^{-1}) and essential oils (1%, 5% and 10%) were tested against the bacterial wilt pathogen *Ralstonia solanacearum* by agar well diffusion method. The hexane, chloroform and methanol extracts showed more or less the same level of antimicrobial activity with a zone of inhibition ranging from 3-9 mm. The essential oils exhibited a zone of inhibition ranging from 3-7 mm.The major constituents of the essential oils were β-myrcene and β-pinene. The results indicated that the rhizome of mango ginger may contain compounds that are toxic to the pathogen. The extracts of mango ginger could be explored further for developing a natural bactericide against *R. solanacearum*.

Keywords: bacterial wilt, essential oils, gas chromatography-mass spectrometry, mango ginger, rhizome extracts

Introduction

Bacterial wilt disease is caused by *Ralstonia solanacearum* (Smith), a soil-borne, rod-shaped gram-negative bacterium that affects hundreds of plant species, including many crops such as tomato, potato, tobacco, pepper, eggplant, banana, cowpea and peanut (Hayward 1991). In India, bacterial wilt of ginger has found a wide distribution resulting in almost 100% yield losses (Dohroo 1991; Mathew *et al.* 1979). *R. solanacearum* could infect many ginger species without wounding and could survive for longer periods which show the potential risk it incurs in ginger growing areas (Paret *et al.* 2006). As ginger is an asexually reproducing plant, there is a lack of genetic variability among the accessions of ginger for disease resistance, which is one of the constraints in its genetic improvement. Resistance breeding in ginger is restricted to germplasm screening (Ravindran *et al.* 2005). The search for the resistance has been extended to other closely related genera in the family, Zingiberaceae such as *Curcuma amada, C. longa, C. zedoaria, C. aromatica, Kaempferia galanga, Elettaria*

cardamomum and Zingiber zerumbet for their response to *R. solanacearum*. The Indian mango ginger (*Curcuma amada* Roxb.) exhibited significant resistance to *R. solanacearum* (Kumar *et al.* 2006; Prasath *et al.* 2011). A study on the factors governing the high level of resistance recorded in mango ginger to bacterial wilt may provide an opportunity for developing bacterial wilt resistance in ginger (Kumar *et al.* 2006).

The Indian mango ginger (*C. amada*) is aherbaceous perennial belonging to the family *Zingiberaceae*. It has a characteristic raw mango flavour which is mainly imparted by car-3-ene and cis-ocimene present in the essential oils (Achut & Bandyopadhyaya 1984). It has antibacterial, insecticidal, antifungal and antioxidant properties (Jatoi *et al.* 2007). An antioxidant compound 'amadannulen' from the rhizome of mango ginger exhibited antimicrobial activity (Policegoudra *et al.* 2007). Amadannulen also showed antibacterial and bactericidal activities. In the present study biochemical analysis was carried out to study the mechanism of resistance of rhizome of mango ginger against *R. solanacearum*.

Materials and methods

Collection of mango ginger rhizomes

Fresh and healthy mango ginger rhizomes were collected from National Active Germplasm Site of ICAR-Indian Institute of Spices Research, Experimental Farm, Peruvannamuzhi, Kerala.

Inoculum production and culture conditions

The virulent strains of *R. Solanacearum* obtained from Division of Crop Protection, ICAR-Indian Institute of Spices Research were cultured on Casamino acid-Peptone-Glucose (CPG) agar medium supplemented with 0.005% (w/v) 2,3,5-triphenyl tetrazolium chloride (TZC) medium at 28°C (Kelman 1954) and the colonies formed were multiplied in CPG broth for 16 hours. The broth was centrifuged and the bacteria were pelleted. The pellet was mixed with sterile water and made up to a concentration of 10^8 cells mL^{-1}.

Preparation of rhizome extract

The rhizomes of mango ginger were washed twice in sterile water, sliced and dried at 50°C for 3 days. The rhizome extract was prepared according to Policegoudra *et al.* (2006) with slight modifications. The dried rhizomes were powdered and 50 g of the powder was sequentially extracted with n-hexane, chloroform and methanol in the order of their increasing polarity at room temperature. All the chemicals used for extraction were of AR grade from Merck Ltd, Mumbai. After each extraction the solvents were filtered and concentrated using a rotary flash evaporator. The yields of extracts were noted 50 µL of the solvent extracts were tested against *R. solanacearum* at different concentrations ranging from 5, 10, 25, 50, 75 mg mL^{-1} and 100 mg mL^{-1}. Dimethyl sulfoxide (DMSO) was used as negative control.

Extraction of essential oil

Essential oil was extracted from 500g of dried and crushed mango ginger rhizomes by hydro-distillation using Clevenger type apparatus. The extracted essential oils were tested at 1%, 5% and 10% concentrations (v/v) against *R. solanacearum*.

Antibacterial activity assay

Antibacterial assay was carried out by agar well diffusion method (Valgas *et al.* 2007). 100 µL of 16 hrs old bacterial culture at OD-0.1 (10^8 CFU mL^{-1}) was spread on the surface of Casein Peptone Glucose (CPG) agar plates. Wells of 6 mm diameter were made with a sterile cork borer. 0.05 mL of extracts was added into the wells and allowed the extracts to diffuse. The control consisted of the DMSO alone and served as the negative control. The plates were incubated at 28°C for 48 to 96 hrs. Inhibition of the bacterial growth was measured in mm.

Statistical analysis

For antibacterial assay test, a completely randomized design (CRD) was used with seven concentrations and three replicates. All the data were expressed as mean of three replicates and

analysed using MSTATC software for analyses of variance (ANOVA).

Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS analysis of the essential oil was performed using a Shimatzu GC-MS QP-2010, Column Rtx-5. Helium gas was used as the carrier gas at a constant flow rate of 1 mL min^{-1}. The injection port temperature was set at 260°C and detector temperature at 250°C. Oven was programmed for 60°C for 5 min; up to 110°C @5°C min^{-1}, 200°C @30°C min^{-1}, 220°C @5°C min^{-1}. For GC-MS detection an electron ionization system with ionization energy of 70 eV, mass range of 60-450 amu and split ratio of 1: 40 was used.The relative percentage of the oil constituents was expressed as percentages by peak area normalization. Compounds were identified by matching the spectral fragmentation pattern of the compound with those stored in the National Institute of Standards and Technology (NIST) & WILEY Library and mass spectra in Adams (1995).

Results and discussion

Plants are constantly exposed to a wide variety of pathogenic microorganisms. Plant diseases caused by bacteria and fungi significantly contribute to the overall loss in crop yield worldwide (Montesinos 2007; Savary *et al.* 2006). Plants have devised various strategies as defence mechanism against pathogens. Pesticides provide an effective control, but their threats to human health and the environment is adverse. Research focused on either introducing resistance into susceptible plants by transferring desired genes from wild relatives by gene transfer techniques or intensification of plant derived bactericides.The present study is investigated the antibacterial activity of mango ginger rhizome extracts. The sequential extraction with hexane, chloroform and methanol yielded 4.2, 0.9 and 2.8 g of extracts respectively. The preliminary screening of all the three extracts showed the same level of antibacterial activity against *R. solanacearum* as indicated by a clear zone of inhibition around the agar well supplemented with extract (Fig. 1 & Table 1). The solvent extracts produced a zone of inhibition ranging from 3-9 mm. The highest inhibition was found in the well supplemented with 0.05 mL of solvent extracts at a concentration of 100 mg mL^{-1}. The inhibitory activity of the three extracts increased with the increase in concentration

Table 1. *In vitro* evaluation of extracts of mango ginger against *R. solanacearum*

Concentration of extracts (mg mL^{-1})	Zone of inhibition (mm)			Mean
	Hexane extract	Chloroform extract	Methanol extract	
100	9.00	9.67	9.00	9.33
75	8.33	8.67	8.33	8.44
50	7.33	7.33	7.33	7.33
25	6.33	6.00	6.00	6.11
10	5.33	5.33	4.67	5.11
5	3.33	4.00	3.00	3.44
Control	0.00	0.00	0.00	0.00
Mean	6.61	6.83	6.44	
			SEd±	CD (P<0.05)
Extracts	NS		0.39	0.83
Concentrations	S		0.45	0.95
E x C	S		0.79	1.65
CV (%)	4.03			

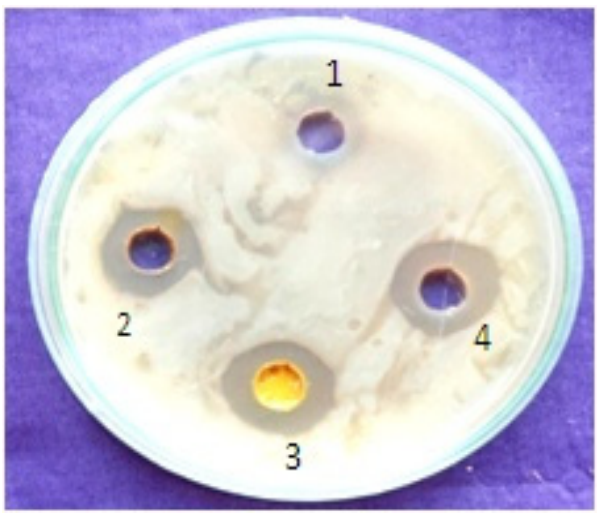
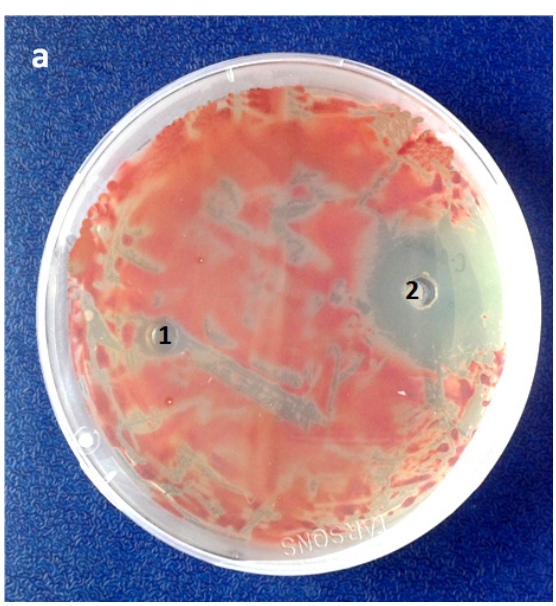


Fig. 1. Petriplate showing zone of inhibition of hexane (75 mg mL^{-1}), chloroform (75 mg mL^{-1}) and methanol (75 mg mL^{-1}) rhizome extracts against *R. solanacearum* (1) Control (DMSO) (2) Hexane extract (3) Chloroform extract (4) Methanol extract

with reference to the control. The method of extraction did not show any significant variation with respect to the zone of inhibition.

Antibacterial activity of free and bound phenolics from mango ginger rhizomes has been reported by Siddaraju & Dharmesh (2007). The antibacterial effect of different extracts like hexane, chloroform, ethyl acetate, acetone and methanol against human pathogen has been reported (Policegoudra *et al.* 2007). The volatile oil from mango ginger rhizome possesses antifungal properties (Singh *et al.* 2002). Difurocumenonol is a novel antibacterial compound that was effective against a wide range of gram-positive and gram-negative bacteria was isolated and characterized from mango ginger rhizome (Policegoudra *et al.* 2007).

Essential oils are potential source of novel antimicrobial agents especially against bacterial pathogens (Mitscher *et al.* 1987). Essential oils from mango ginger rhizome exhibited antibacterial activity at 1%, 5% and 10% (v/v) against *R. Solanacearum* (Fig. 2). The antibacterial activity of the essential oils increased with the increase in concentration. GC-MS analysis identified the chemical constituents of the essential oils. Myrcene and



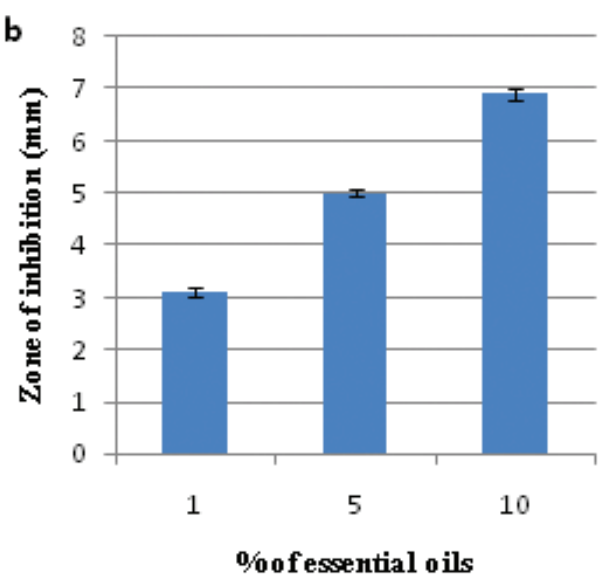


Fig. 2a. Petriplate showing the zone of inhibition essential oil of mango ginger against *R. Solanacearum* (1. Control; 2. Essential oil)

Fig. 2b. Inhibitory activity of essential oils against *R. solancaearum*

pinene were the major constituents of the essential oils (Table 2). It was earlier reported that myrcene and pinene are responsible for antifungal activity against the wide range of fungi, *viz*., *Curvularia palliscens*, *Aspergillus niger, A. terreus, Fusarium moniliforme* and *F. falcatum* (Policegoudra *et al*. 2007). The results show that the extracts from mango ginger rhizomes possessed antibacterial activity against *R. solanacearum*, confirming the great potential of bioactive compounds present in it. It is concluded that the essential oil and extracts from mango ginger could be explored further for developing a novel natural bactericide to manage bacterial wilt caused by *R. solanacearum*.

In the present work volatile oils and three solvent extracts (hexane, methanol and chloroform) from the rhizome of mango ginger were evaluated *in vitro* against the bacterial wilt pathogen. All the three solvent extracts and essential oils possessed antimicrobial properties. GCMS analysis revealed that the major components of the essential oils were myrcene and pinene. As many terpenes are vital components of plant resistance to biotic and abiotic stresses (Kang *et al.* 2010; Rodriguez *et al*. 2011), in addition to myrcene and pinene, it is also possible that the trace/minor components present in essential oils, such as perillene, caryophylline oxide, camphene, limonene, terpineol etc. might be involved in some type of synergism with other active components of essential oil. The study concludes that the constituents of the rhizome of mango ginger might be inhibiting the entry of bacteria through the rhizome.

Table 2. GC-MS analysis on composition of dried mango ginger rhizomes

Constituent	Area (%)
β-Myrcene	38.00
β -pinene	10.28
Perillene	3.74
Caryophyllene oxide	2.65
Camphene	1.98
Cis-ocimene	1.77
D-limonene	0.94
α Terpineol	0.91
E-Caryophyllene	0.90
n-Hexadecanoic acid	0.72
1,8-Cineole	0.65
Camphene	0.64
Ar-Turmerone	0.52
Linalool	0.46
Geranial	0.29
2-Nonanone	0.38
Trans-ocimene	0.33
Cymene	0.25
Trans-Pinocarveol	0.25
Myrtenal	0.21
Borneol	0.21
Myrtenol	0.20
Perillyl alcohol	0.20
z-Citral	0.17
Curlone	0.16
Turmerone	0.16

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