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ORIGINAL PAPER

Effect of Essential Oils from *Lippia geminata* and *Cymbopogon jwarancusa* on In vitro Growth and Sporulation of Two Rice Pathogens

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Abstract Sheath blight and brown spot disease of rice caused by *Rhizoctonia solani* and *Bipolaris oryzae* causes significant yield loss in rice production worldwide. The present study was undertaken to examine the effect of two essential oils (EOs) from *Lippia geminata* and *Cymbopogon jwarancusa* on in vitro growth and sporulation of these two pathogens. The fungal radial growth was inhibited at very low concentration (25 ppm) of the EOs. Similarly, fungal spore production was also inhibited up to ≥80% at 500 ppm of EOs. However, fungal sporulation was completely retarded at 1,000 ppm of *L. geminata* EO. Very low oil concentrations (10 ppm) accelerated the radial growth (0–5 mm) and spore germination (3.5–8.5%) of the pathogens. At higher oil concentrations, the mycelial growth and spore productions were completely inhibited. The IC_{50} value of the EO of *C. jwarancusa* was 365.45 and 336.74 ppm and for *L. geminata,* it was 420.16 and 481.47 ppm against *B. oryzae* and *R. solani,* respectively. GC–MS analysis of the oils showed 54.36% piperitone and 30.86% α-phellandrene as major compounds in *C. jwarancusa* whereas 25.9% geranial and 14.6% neral in *L. geminata* oil. Essential oils from *Lippia geminata* and *Cymbopogon jwarancusa* appear to be good candidates for the in vitro control of these two rice pathogens and can be successfully utilized in management strategies of pathogens in appropriate formulation.

Keywords Antifungal · Essential oil · Fungal sporulation · *Rhizoctonia solani* · *Bipolaris oryzae*

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Introduction

Rice (*Oryza sativa* L.) is an immensely important crop consumed by more than half the world's population. Cultivation practices adopted in different parts of the globe also differ widely depending upon the climatic conditions, soil properties, availability of water, fertilizer level, crop variety etc. Because of all those factors including cultivation practices during different seasons of the year, several diseases infect the crop resulting in extensive damage to the grain and straw yields. In Assam (India), this major cereal crop occupies an area of 2.7 million hectares [1].

Sheath blight of rice caused by *Rhizoctonia solani* is widely distributed in rice growing regions and has now been recognized as the "most important" disease in some rice growing regions and countries [2]. It was first reported from Gurdaspur by Parcer and Chahalin in India [3]. It causes substantial yield loss of about 5.2–50% as a whole whereas this inflicts around 5–35% grain loss in Assam. The disease was common in 50–66% of rice fields and yield reductions up to 20% may be induced when sheath blight epidemics develop and reach to the uppermost leaves of the plants [4]. Due to favorable temperature and humidity during March to July, the disease appears more severe in autumn rice in the region. Because of the

significant economic importance, sheath blight has received great attention from plant pathologists and rice growers in their disease control practices [5, 6]. The pathogen survives for a long period in the soil and rice stubble in the form of viable sclerotia.

Brown spot of rice caused by *Bipolaris oryzae* (formerly *Helminthosporium oryzae* Breda. De Hann) is also a serious disease occurring in almost all the rice growing areas throughout the world and is responsible for losses in yield of up to 90% [7]. In India, the disease was first recorded in 1922 and presently it is found as an epiphytic form from all the Southern parts (Andhra Pradesh, Kerala, Tamilnadu and Karnataka) and eastern states of India [8]. When the disease appears, the grains become shriveled and discolored, the plants are stunted and sometimes all the leaves wither and the whole field presents a highly characteristics burnt or scorched appearance.

Plant essential oil (EO) is widely used as a flavoring agent by the food industry and is regarded as safe by the Food and Drug Administration of the United States [9]. These novel plant based bioactive compounds can be successfully used against the foliar or soil borne pathogens or as an antifungal agent in a safe manner. Tegegne et al. [10] proved that the aerial part crude extract of *Agapanthus africanus* possessed sufficient in vivo antifungal activity against plant pathogens and also increased the yield of peas and sorghums. In contrast, chemical treatment is one of the classical methods of disease management by seed dressing, foliar sprays as well as soil application of various fungicides. Most of the fungicides like benomyl, carbendazim, chloroneb, captafol, mancozeb, zineb, edifenphos, IBP, thiophanate, CGA 64250, carboxin etc., have been found effective for the control of fungal diseases under field conditions. Among these, benomyl, carbendazim, edifenphos and IBP have been extensively used as fungicides in India. However, these synthetic pesticides have deleterious effects on the health of consumers, fungicide resistance by pathogens, non-target injury, etc. Therefore, there is a demand for the development of new, safe, biodegradable alternative natural fungicides having maximum efficacy with minimal environmental impact and danger to consumers.

Little information is available on disease management strategies against sheath blight and brown spot disease by bioactive plant metabolites in the field other than spraying with fungicides. However, no rice cultivar has been found to be completely resistant to the sheath blight pathogen of rice [2]. Therefore, the study was taken to find out in vitro antifungal activity of EOs for newer bioactive compounds which may have possible application in the field after appropriate formulation. The EO of *Cymbopogon jwarancusa* and *Lippia geminata* H.B.K syn *L. alba* Mill containing novel bioactive compounds was tested against both the rice pathogens i.e. *B. oryzae* and *R. solani* [11].

Materials and Methods

The Study Area and Disease Survey

The study was carried out at the North East Institute of Science and Technology (NEIST), Jorhat, Assam (India), the coordinates of the district are 91′40′16″ E longitude and between 25′40′ and 26′20′N latitude. The climate is warm humid with an annual average rainfall of about 2,000 mm and rainy season begins in the early part of April and continues till October. During the study the maximum temperature did not exceed 40 °C and winter temperature did not fall below 5 °C. The seasonal rice cultivation started in April–August and the harvest was in November–December. The disease survey was conducted in the farmer's cultivated field during the cultivation period and infected leaves were isolated.

Isolation of Disease Causal Organisms

The infected diseased leaves were collected from farmer's rice fields in the Jorhat districts of Assam, India. Isolation was done in a Laminar-air-flow chamber under aseptic condition. Infected host tissues were selected from the advancing margin of the lesion, cut into small pieces, placed in mercuric chloride ($HgCl_2$) solution (1:1,000) for 1–1½ min; then washed with sterile distilled water three times. The surface sterilized pieces were put on a Potato Dextrose Agar (PDA) plate and incubated at 27 ± 2 °C. After 3–5 days, the fungal growth associated with the pieces was examined and aseptically transferred to PDA slants and PDA plates. Vegetative and reproductive structures were examined under a light microscope (Olympus Binocular Model KIC22781) for identification of the microorganism according to Subramanian [12]. Confirmation of the pathogens were done using Koch Postulates.

EOs and Identification of Major Compounds

Fresh leaves of *L. geminata* and *C. jwarancusa* were collected from the Experimental Garden of NEIST, Jorhat for isolation of EOs. The fresh leaves were subjected to hydrodistillation in a Clevenger's apparatus for four hours for the collection of the oils. EOs were collected in glass vials after removing water traces by sodium sulfate and stored at 4 °C for further study. The major compositions of the EOs were analyzed by GC–MS.

A Chemito 8510 GC instrument equipped with a data processor was used for GC analysis. A BP-5 wide-bore capillary column (30 m × 0.53 mm i.d., 1.0 μm film thickness) was used for sample components (sample size 0.03 μl, measured using a Hamilton GC syringe of 1.0 μl

cap.). Hydrogen gas was used as the carrier at 5 ml/min flow rate and 20 p.s.i. inlet pressure; split ratio, 1:20. The GC column oven temperature was 70–210 °C at a rate of 2.5 °C/min, with a final hold time of 5 min. Both injector and detector (FID) temperatures were maintained at 230 °C. GC–MS analysis was carried out on a Trace DSQ MS (Thermo Electron Corporation), using a BP-5 capillary column (30 m × 0.25 mm i.d., 0.5 μm film thickness); the carrier gas (helium) at a flow rate of 1 ml/min; split ratio 1:20. The column temperature was 65–210 °C (10 min hold) at 3 °C/min. Mass spectra were recorded in the range 50–450 amu, operating at 70 eV and the ion source temperature was maintained at 200 °C. Oil constituents were identified by matching the retention times with standard reference compounds and also by matching the mass spectra fragmentation pattern with the NEIST-Wiley Mass Spectra Library stored in the GC–MS library.

EO and Radial Growth of Pathogens

From each sample of EO, different concentration of the oil viz. 10, 50, 100, 150, 200, 250, 500, 750, and 1,000 ppm were prepared in ethylene glycol. After gelation of the medium, a 1-cm diameter specimen of the 8-day old fungal culture was placed on the PDA media in the petri plates with the help of a sterile cork borer and then incubated at 27 ± 2 °C. After 8 days of incubation, radial growth was observed and the diameter of each fungal mat was measured. The diameter of the fungal colonies (mm) was measured at two-day intervals and the percentage inhibition of radial growth calculated by the formula Dc-Dt/Dc × 100 where Dc and Dt is the diameter (mm) in the control and treated plates, respectively.

EOs and Sporulation of the Pathogens

Spores from the colonies incubated for 6–10 days (until spore formation) of *B. oryzae* and *R. solani* previously exposed to both the oil enrichment (at 10, 100, 250, 500, 750, and 1,000 ppm) were collected by adding 5 ml sterile water containing 0.1% v/v Tween 80 (for better spore separation) to each Petri dish and rubbing the surface three times with a sterile L-shaped spreader. The suspension was collected and then centrifuged at room temperature at 2,000*g* for 5 min. The supernatant was discarded and the pellet re-centrifuged until 1 ml of the highly concentrated spore solution remained. A hemocytometer slide was used to count spore concentration.

Statistical Analysis

All experiments were replicated four times. The data were recorded in a Microsoft Excel spreadsheet and analyses were done by the Statistical Analysis System (SAS). Standard error and significant differences between values were determined using Duncan's multiple range test ($p < 0.05$), following one-way ANOVA. The graphs and the other figures are presented with the help of the MS Excel program and statistical software 'Origin 7.5'.

Results

Disease Survey

The disease survey was carried out during February–March in the experimental sites at the Regional Agricultural Research Station, Titabar and Assam Agricultural University, Jorhat during December–January. It was observed that there were localized lesions developed on leaves and stems consisting of dead and collapsed cells on the host. Collected diseased seedlings and plants were examined. Plant residues of *Sali* and *Bodo* (two indigenous rice crop) rice containing sclerotia were collected and examined. The infected leaves of sheath blight disease were light brown lesions on the coleoptiles and seminal roots, circular lesions with a reddish brown margin and a grey center on the leaves. Lesions on the leaf sheaths were noticed on plants affected by brown spot disease. Symptoms appeared on leaves were irregular patches, spread rapidly to the stem, brown to dark brown lesions on stems covered by the leaf sheath, in the case of plants affected by sheath blight disease. In the microscopic examination, the pathogen was identified as *R. solani*.

The pathogen isolated from the brown spot disease was examined under the microscope. The mycelia were brown, septate, cellular or intercellular; conidiophores septate, darker at the base, slightly bent; conidia slightly curved with a bulge in the middle and tapering at ends, brownish when mature; 5–10 celled. The pathogenic organism was identified as *B. oryzae* [8]. The mycelia of the sheath blight pathogen were highly variable in appearance depending on their age; initially colorless hyphae then becoming yellowish to deep brown in later stages. Sclerotia were irregular in shape, black, varying in size [12].

Chemical Compositions of EOs

The major chemicals components of *C. jwarancusa* were piperitone (54.36%), α-phellandrene (30.86%), limonene (3.4%), *p*-Cymene (1.57%), etc. The species belongs to Piperitone/α-Phellandrene chemotype. The components of the oil were somewhat comparable to the components identified by Singh and Pathak [13] and Saeed et al. [14] (Table 1). The major components of the *L. geminata* oil were geranial (25.9%), neral (14.6%), myrcene (12.7%),

Table 1 Major chemical compositions of *C. jwarancusa* and *L. geminata* EO

Sl no.	Major compounds	*C. jwarancusa*			*L. geminata*		
		Present study	Singh and Pathak [13]	Saeed et al. [14]	Present study	Sousa et al. [16]	Shukla et al. [17]
1	Geranial	–	–	–	25.9	39.602 (6.31)	22.21
2	Neral	–	–	–	14.6	29.136 (4.09)	14.2
3	α-Phellandrene	30.86	14.73 (8.2)	–	1.0	–	–
4	Myrcene	–	–	0.02	11.7	4.05 (2.69)	7.17
5	Limonene	3.4	2.02 (0.74)	–	3.7	1.276 (0.79)	0.25
6	*β*-Pinene	–	0.06 (0.01)	0.05	7.2	–	–
7	*p*-Cymene	1.57	0.73 (0.43)	0.05	–	–	–
8	Caryophyllene oxide	–	0.22 (0.06)	–	6.1	–	–
9	*δ*-Cymene	1.57	–	0.05	–	–	0.40
10	Piperitone	54.36	71.09 (8.6)	65.4	–	–	–
11	*β*-Guaiane	–	–0	–	3.6	–	–
18	Geraniol	–	–	0.04	4.5	2.982 (1.21)	–
19	Nerol	–	–	–	5.5		1.17
20	Linalool	–	–	0.03	3.8	16.048 (10.05)	1.59
21	*β*-Guaiane	–	–	–	2.1	–	–
22	*β*-Caryophyllene	0.82	0.67 (0.04)	1.2	1.8	3.074 (0.81)	6.17
23	Geranyl acetate	–	–	–	–	–	3.09
24	(−)Elema-1,3,11(13)-trien-12-ol	–	–	–	–	–	4.28
25	1-Hepten-3-ol	–	–	–	–	–	4.46
26	Methyl heptenone	–	–	–	–	–	2.84
27	*trans*-Piperitol	–	1.48 (0.23)	0.07	–	–	–
28	Terpinolene	–	0.50 (0.05)	0.03	–	–	–
	Total compounds identified by the author (%)	92.58	95.36 ($N = 3$)	94.0	91.5	99.162 ($N = 5$)	91.05

Data in parenthesis are standard errors

α-pinene (9.2%) and caryophyllene oxide (6.1%) etc. (Table 1). The major composition of oil indicates the species belongs to geranial/neral chemotype [15]. However, this composition of the essential oil was different in the studies reported by Sousa et al. [16] and Shukla et al. [17].

Antifungal Activity of the EOs

The antifungal activity of the EO was tested measuring the radial growth of the fungal colony against different concentrations of the oil (Figs. 1, 2). The radial growths of fungal colonies were measured on each alternative day (Figs. 3, 4). Oil of *C. jwarancusa* and *L. geminata* were effective at a very low concentration (100 ppm). However, the lowest concentration of oil (10 ppm) accelerated the growth of both the pathogen compared to control against both the fungi tested. The MIC of the *C. jwarancusa* was 750 ppm for both the pathogens whereas the MIC of *L. geminata* was 1,000 ppm for *R. solani*. The growth of the fungal pathogens was maximum between days 4 and

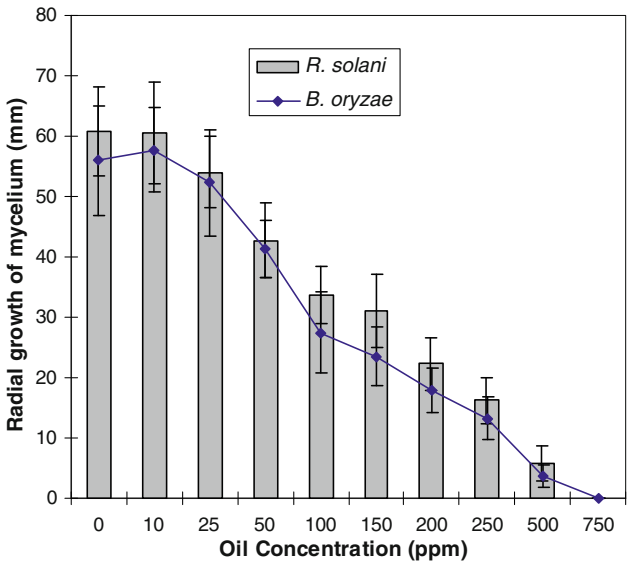

Fig. 1 Effect of *Cymbopogon jwarancusa* EO on the radial growth of the fungal mycelium on PDA (significant difference between the effects of oil at $p < 0.05$, ANOVA test)

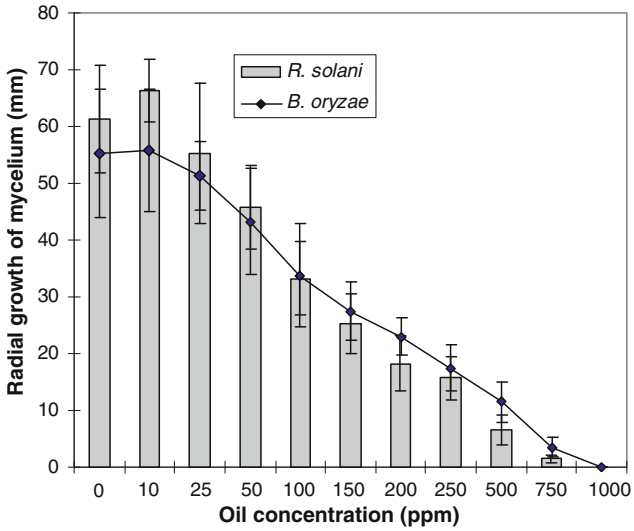

Fig. 2 Effect of *Lippia geminata* EO on the radial growth of the fungal mycelium on PDA (significant difference between the effects of oil at $p < 0.05$, ANOVA test)

6 of incubation in the control and low concentrations of the EO. However, the growth rate was linear in the 500 ppm concentration. The various chemical components presents in the EOs were responsible for the antimicrobial activity against the fungal pathogens [11].

EOs and Sporulation of the Pathogens

Fungal sporulation on PDA was significantly ($p < 0.05$) reduced by the EO of *C. jwarancusa* with spore reduction of up to 71% (±10.7) in *B. oryzae*, 73.5% (±13.6) in *R. solani* and which was 63% (±9.2) and 54.0% (±7.4) reduction against *L. geminata* oil at 500 ppm concentration (Figs. 5, 6). Moreover, spore production was completely inhibited at 750 ppm and 1,000 ppm for both the pathogens tested. The IC_{50} value of the EO of *C. jwarancusa* was 365.45 and 336.74 ppm and for *L. geminata,* it was 420.16 and 481.47 ppm against *B. oryzae* and *R. solani.* It was also observed that at a very low concentration of oil (10 ppm), the spore production of *R. solani* increased by 7.9% (*C. jwarancusa)* and 5.0% (*L. geminata*), respectively. However, the increased percentage of conidia was lower than that of *B. oryzae* (8.5 and 3.5%) against the oils at the same concentration. The variations in antimicrobial activity of EOs may be due to their different chemical compositions.

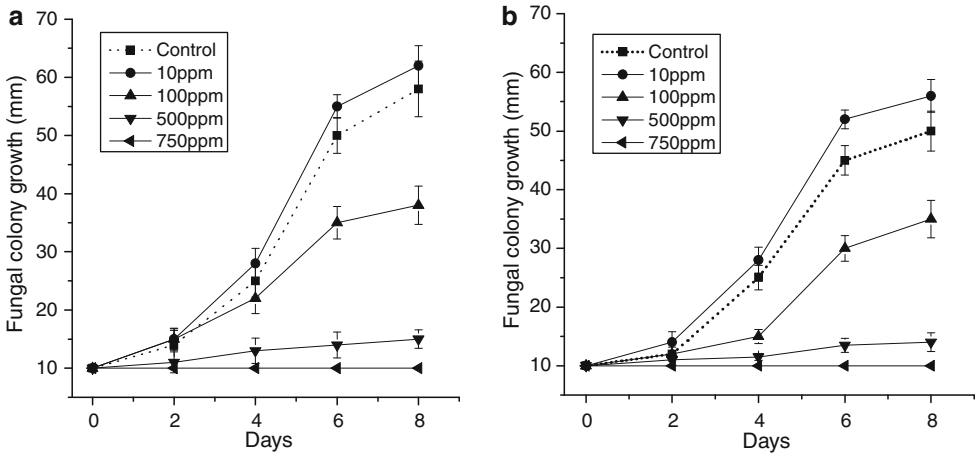

Fig. 3 Effect of *Cymbopogon jwarancusa* essential oil on colony growth of *B. oryzae* (**a**) and *R. solani* (**b**) on PDA at 27 ± 2 °C

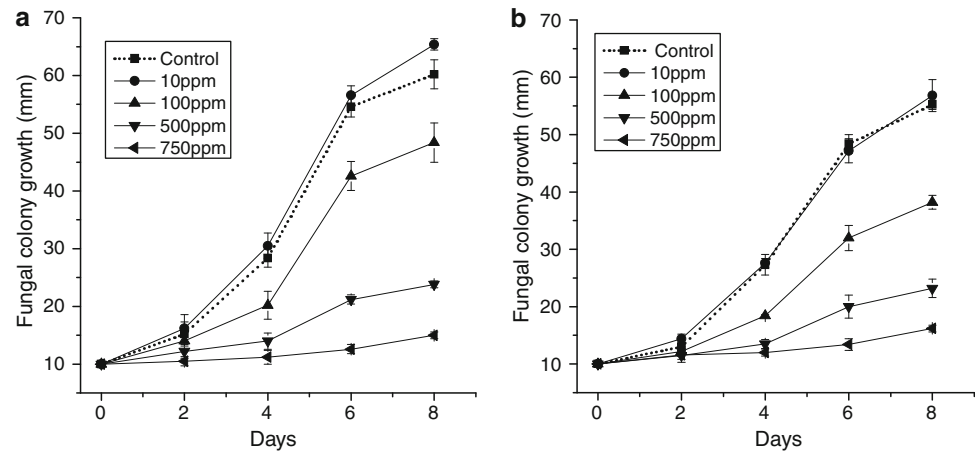

Fig. 4 Effect of *Lippia geminata* essential oil on colony growth of *B. oryzae* (**a**) and *R. solani* (**b**) on PDA at 27 ± 2 °C

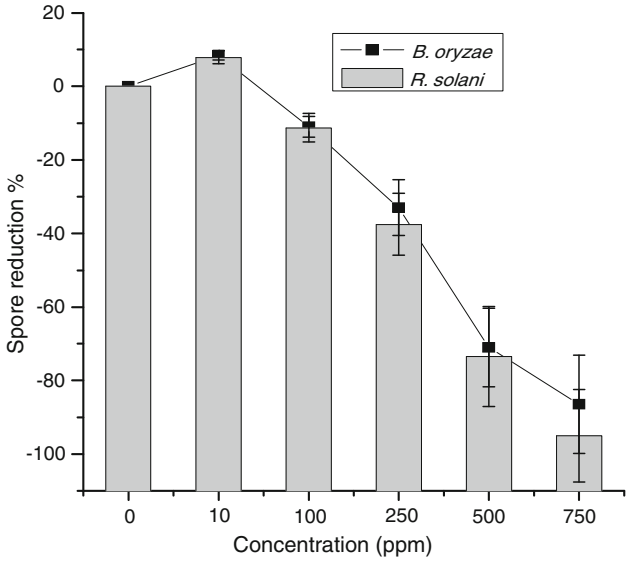

Fig. 5 Effect of *Cymbopogon jwarancusa* EO on conidia reduction of *B. oryzae* and *R. solani* on PDA at 27 ± 2 °C

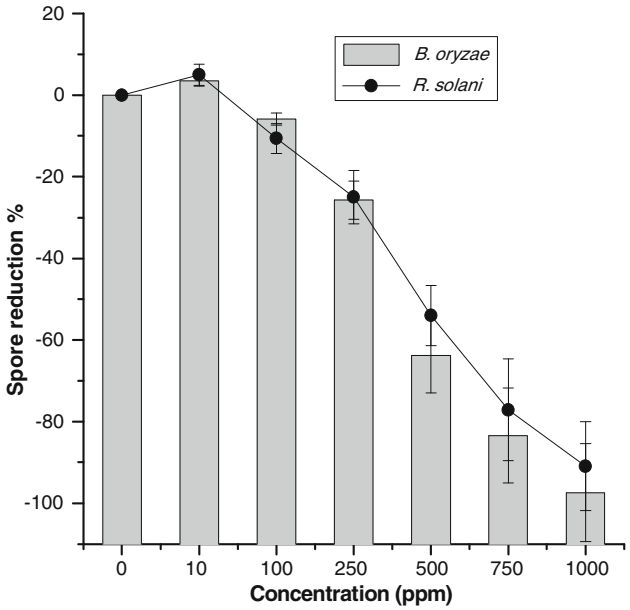

Fig. 6 Effect of *Lippia geminata* EO on conidia reduction of *B. oryzae* and *R. solani* on PDA at 27 ± 2 °C

Discussion

Plant-based EOs have the ability to control some the of the soil born plant pathogens in the nursery independent of soil texture having a promising potential for controlling soil born plant pathogens [9, 18]. In this investigation, both rice pathogens were very sensitive to different concentrations of EOs. Singh et al. [19] found that the growth of *B. oryzae* was completely inhibited by *C. oliveri* and *Trachyspermum ammi* oil at 750 ppm and *C. martini* oil at 700 ppm. The growth of *Aspergillus* sp. (storage fungi) was significantly inhibited (50–100%) by the EOs of *Amomum subulatum, Aegle marmelos, Ageratum houstonianum, Alpinia galangal, L. alba*, *Curcuma longa*, *Artemisia vulgaris* and *Salvia plebeian* at 5,000 ppm which did not have any adverse effect on seed germination of *O. sativa* [20]. Kurita et al. [21] have shown that geranial and neral acts as fungicidal agents as they are able to form a charge transfer complex with an electron donor to fungal cells, which results in fungal death. The US National Toxicology Program (NTP) reported that citral (Geranial and neral) did not cause cancer in male or female rats receiving 4,000 ppm (∼3.56 mg/ml) citral in the feed for 2 years. The acceptable daily intake is 5 mg citral/kg body weight which is Generally Recognized As Safe (GRAS) status in the USA.

The leaf extracts of *L. alba* and *Chenopodium ambrosioides* were found to be toxic against *Pythium aphanidermatum* and *P. debaryanum,* organisms causing damping-off in tomato [22]. Both the EOs were fungitoxic but not phytotoxic. Even as a vapor treatment it was very effective against fungal growth and sporulation in a small amount of oil. Seed soak with *L. alba* oil exhibited 88.9 and 71.3% reduction in damping-off diseases of tomato when shown in soil infested with *P. aphanidermatum* and *P. debaryanum,* respectively. However, the application of bacterial antagonist or other such biocontrol agents was not at all successful with in vivo application [23]. The formulation of *Streptomyces* sp. reduced the disease intensity under greenhouse conditions which had no activity under field conditions against *R. solani* [24].

Handique and Singh [25] observed that the EO of lemongrass (mutant strain LM-81) at 100 ppm decreased the growth (67%) of *R. solani* and *Sclerotinia sclerotiorum.* EO of *Tanacetum annuum* exhibited complete inhibition of mycelial growth of four fungal pathogens viz. *Botrytis cinerea, B. oryzae, Pyricularia oryzae* and *Verticillium dabltae* at 5,000 ppm [26]. Antifungal activity of EO of *Salvia pomifera* and its main components (α- and β-thujone) were tested against six phytopathogenic fungal strains. The hydrocarbon and oxygenated fractions of the oil of *Salvia pomifera* were assessed against *R. solani* and *S. sclerotiorum* by Pitarokili et al. [15]. The EO was found to be fungistatic at 1,000 μL/L for both the fungi, whereas its oxygenated fraction showed fungicidal activity at 2,000 and 1,000 μL/L, respectively. They also mentioned that the hydrocarbon fraction had a weak effect on the test organisms and oxygenated monoterpenes (α- and β-thujone) exhibited a moderate fungistatic effect on both *R. solani* and *S. sclerotiorum.*

The in vitro antifungal activity of pure citral, *C. citratus* and *L. rehmannii* essential oil was superior

Table 2 Antifungal activity of selected plant essential oils against *R. solani* and *B. oryzae*

Certain antifungal essential oils	MIC (/*IC_{50}) value against pathogens		References
	R. solani	*B. oryzae*	
L. geminata	0.48 μL/mL*	0.42*	Present study
C. jwarancusa	0.75 μL/mL	0.75 μL/mL	Present study
	0.337 μL/mL*	0.366 μL/mL*	
Metasequoia glyptostroboides	1000 μg/mL	–	[29]
Citrus reticulata	0.2 ml/100 mL	>0.2 mL/100 mL	[18]
Lippia rehmannii	20 μL/L	–	[30]
Cymbopogon citratus	50 μL/L	–	[30]
Chenopodium ambrosioides	–	>100 μg/mL	[31]
Cymbopogon citratus	–	0.60 μL/mL	[32]
Ocimum gratissimum	–	0.80 μL/mL	[32]
Thymus vulgaris	–	1.00 μL/mL	[32]

Table 3 Antifungal activity of *C. jwarancusa* and *L. alba* syn *L. germinata* essential oils against some fungal pathogens/contaminants

Fungal pathogens/contaminants	MIC (/*IC_{50}) value of essential oils (μL/mL)		References
	C. jwarancusa	*L. alba*	
B. oryzae	0.37 μL/mL*	0.42 μL/mL*	Present study
R. solani	0.34 μL/mL*	0.48 μL/mL*	Present study
Aspergillus flavus	–	>500 μg/mL	[33]
Aspergillus favus	–	>1.0 μL/mL	[17]
Aspergillus fumigatus	–	>500 μg/mL	[33]
Aspergillus fumigatus	–	1.0 μL/mL	[17]
Candida parapsilosis	–	>500 μg/mL	[33]
Alternaria alternata	–	>1.0 μL/mL	[17]
Aspergillus glaucus	–	1.0 μL/mL	[17]
Aspergillus niger	–	>1.0 μL/mL	[17]
Aspergillus shydowi	–	0.75 μL/mL	[17]
Aspergillus terreus	–	>1.0 μL/mL	[17]
Cladosporium cladosporioides	–	>1.0 μL/mL	[17]
Curvularia lunata	–	>1.0 μL/mL	[17]
Fusarium graminearum	–	>1.0 μL/mL	[17]
Fusarium nivale	–	1.0 μL/mL	[17]
Fusarium oxysporum	–	>1.0 μL/mL	[17]
Penicillium italicum	–	>1.0 μL/mL	[17]
Rhizoctonia solani	–	1.0 μL/mL	[17]
Rhizopus stolonifer	–	0.75 μL/mL	[17]
Trichoderma spp.	–	0.75 μL/mL	[17]

(MIC $\leq$ 100 μL/L) to *C. jwarancusa* and *L. geminata* against *Fusarium oxysporum* and *R. solani* although less effective against *Lasiodiplodia theobromae, Colletotrichum gloeosporioides, Alternaria alternata, Penicillium digitatum etc.* (Tables 2, 3). The in vitro growth of *R. solani, F. solani* and *Colletotrichum lindemuthianum* was decreased by 60% at a concentration of 1,600 ppm when treated with *Myrtus communis* EO [27]. The EOs of *Acorus calamus* and *Hedychium spicatum* also inhibited the growth and development of seed-borne pathogens of rice viz *F. moniliforme* [*Gibberelli moniliformis*] and *B. oryzae* [*Cochliobolus miyabeanus*] [28]. However, the EOs of *C. jwarancusa* and *L. geminata* were more effective against Brown spot and Sheath blight disease pathogens under in vitro conditions. The application of plant metabolites in soil and foliar treatment significantly inhibited the

incidence of diseases and also increased the yield. All the above data strongly suggest that these two EOs can successfully be used in controlling rice pathogens.

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