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PLANT PATHOLOGY

Antivirulence activity of essential oils against X*anthomonas oryzae* pv. *oryzae* causing bacterial blight of *Oryza sativa*

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ABSTRACT

The majority of bacterial diseases currently being treated use the traditional antibiotic therapy which gives way to the development of multidrug-resistant bacteria. The seriousness further increases in the microbes which form resilient biofilms. Therefore, researchers around the globe are working towards the development of new bactericides which do not impose the above conditions. The present investigation was thus carried out with the aim to screen natural essential oils from different plant parts against *Xanthomonas oryzae*, causal organism of leaf blight disease in rice. The results revealed decrement in biofilm formation at 1000 ppm in 9 oils out of 15 without considerable decrease in total colony forming units. However, the motility traits viz. swimming was maximally reduced in case of calamous and cajeput oil while swarming was decreased significantly in jatamansi and costus root oil. The other virulence traits like protease and exopolysaccharide responsible for the pathogenecity of the bacterium was maximally reduced in calamous and jatamansi oils. However, maximum decrease in lipase was observed in case of costus root oil. Overall, the results highlight potential anti virulence property of calamous, costus and jatamansi oil which can be in future used for the development of potential bactericide against *X. oryzae.*

Highlights

- Isolation of essential oils from different plant parts collected from CSIR-CIMAP farm and adjoining areas.
- Test for anti- biofilm property of the isolated essential oils against *Xanthomonas oryzae*.
- Study of the effect of selected essential oils on motility and cell wall degrading enzymes.

Keywords: Biofilm, cell wall degrading enzymes, crop protectants, motility

In spite of the considerable progress in plant disease management approaches, our worldwide food supply is still at risk by a massive amount of pests and pathogens. Among the diverse range of pathogens, bacteria causing serious plant diseases are the main stake holders restraining the crop production globally. In addition, as per the reports of Tilman *et al.* (2002) the worldwide demand for food grain will double by 2050 which will further build up the pressure on both researchers and farmers to save the starving population.

Currently the diseases are generally managed to some extent by hazardous chemicals, pesticides and antibiotics which come with numerous side effects. Keeping in mind the extensive public apprehensions for long duration health and effect on environment of synthetic chemicals; researchers around the globe have started looking for safe alternatives. One such approach is disarming the pathogen's virulence potency and biofilm formation capacity by the use of plant based molecules or extracts (Upadhyay *et al.* 2014).

Plants are reported to be well loaded with an array of bioactive molecules and thus are being

looked upon for the search and development of original antivirulence compounds. Additionally, since conventional antimicrobial agents principally manage the disease by inhibiting the cell growth thereby increasing the chance of bacterial drug resistance; essential oils (EOs) represent a potential group of alternative crop protectants. EOs adopt multiple mechanism of action targeting critical steps required for pathogenicity like inhibition of biofilm formation, toxin production and other virulence factors (Sanchez *et al.* 2016). Previously promising effect of essential oils as antimicrobial agents owing to the presence of different classes of plant secondary metabolites like terpenoids and phenolic acids (Helander *et al.* 1998; Silva *et al.* 2017) have been shown by many researchers.

Xanthomonas oryzae, is one of the most ravaging and destructive bacterial plant pathogen limiting rice production especially in South East Asian countries (Gnanamanickam *et al.* 1999). For inciting the disease the bacterium produces a range of factors like exopolysaccharides (EPS) thereby forming resistant biofilm along with many extracellular cell wall degrading enzymes (Lee *et al.* 2005; White *et al.* 2009). A capacious body of evidence exists describing the potential antimicrobial activities of essential oils and their active components, without investigating their effectiveness against bacterial virulence. Therefore, keeping in view the above lacuna in mind the present investigation was carried out with the aim to screen different essential oils present in Indian subcontinent for their anti virulence and anti biofilm forming activities again *X. oryzae*.

MATERIALS AND METHODS

Collection of plant materials and essential oils extraction

Plant specimens were collected from different locations of CSIR-CIMAP, Lucknow, India campus along with the adjoining areas. Sampling tissues selected and concentration used for screening is shown in Table 1. Samples brought to the laboratory were thoroughly washed before oil extraction. EOs were extracted by hydrodistillation using a Clevenger-type apparatus method. Hydrodistillation was carried out in a 1 L round bottom flask with 100 g of plant material and 250 ml of boiling water for 180 min. The EOs was previously saturated with NaCl and further dried using anhydrous sodium sulphate (Cassel *et al.* 2009).

Bacterial growth conditions

The *X. oryzae* AS29 strain was grown at 28 ± 2 °C on Luria Bertani (LB) agar medium. For antibiofilm

Table 1: List of various essential oils screened for anti-biofilm activity against *X. oryzae* AS29. Mean value is an average of three replicates with ± standard error (SE). Tukey's multiple comparison test at a level of 5% of probability ($P \leq 0.05$) was applied. The averages followed by the same letter do not differ statistically between themselves. "+" represents fair, "++"represents potent, and "–"represents no activity

Sl. No.	Name of oil	Code	Plant species	Source	500 ppm	1000 ppm
1	Calamous oil	ST21	*Acorus calamus*	Root	-	++
2	Nar Kachur oil	ST22	*Curcuma zedoaria*	Rhizomes	-	++
3	Olibanum oil	ST23	*Boswellia carteri*	Oleo gum resin	-	+
4	Galangal oil	ST24	*Alpina galanga*	Rhizomes	-	++
5	Thuja oil	ST25	*Thuja occidentalis*	Leaves	-	-
6	Eucalyptus oil	ST26	*Eucalyptus globulus*	Leaves	-	-
7	Carrot seed oil	ST27	*Daucus carota*	Seeds	-	+
8	Cajeput oil	ST29	*Melaleuca leucadendron*	Leaves	-	++
9	Davana oil	ST30	*Artemisia pallens*	Leaves	-	+
10	Juniper berry oil	ST31	Juniperus communis	Berries	-	++
11	Costus root oil	ST32	*Saussurea lappa*	Root	-	++
12	Jatamansi oil	ST33	Nardostachys Jatamansi	Rhizomes	-	++
13	Angelica root oil	ST34	*Angelica archangelica*	Roots	-	++
15	Barmi oil	ST35	*Abies wabiana*	Leaf	-	-
15	Kulanjan oil	ST36	*Alpina galanga*	Root	-	++

and other experiments the overnight culture was prepared by inoculating approximately 1 ml LB broth with pure single colony of bacterium taken from LB plates. The culture was incubated overnight at 28 ± 2 °C with 120 rpm shaking. Inoculum was prepared by diluting overnight grown cultures in sterile distilled water (SDW) to approximately 10^8 CFU ml^{-1}. These culture suspensions were further diluted with SDW as required.

Biofilm formation and colony forming units (CFU)

The effect of various EOs on *X. oryzae* AS29 biofilm formation was determined by measuring the biofilm formation (Coffey and Anderson 2014). To carry out CFU and biofilm analysis, 0.1 ml XooAS29 strain (OD_{600} nm 0.4) was added to 1 ml of respective growth medium with varying concentrations of various EOs (500 and 1000 ppm prepared in 10 % dimethyl sulfoxide; DMSO) with and without shaking for 24 h at 28 ± 2 °C, respectively. For CFU, the culture turbidity was measured at OD_{600} using a spectrophotometer (Microplate reader, Biotek, U.S).

For biofilm development, the planktonic cells in the eppendroff tubes were decanted and the tubes were rinsed twice with SDW. The surface attached cells in the eppendroff tubes were stained with 1 ml of 0.01% crystal violet (CV) solution. After 1 h, the excess CV solution was removed and the stained cells were dissolved with 1 ml DMSO. The biofilm development was measured by calculating the intensity of CV in DMSO at OD_{595} using a spectrophotometer (Microplate reader, Biotek, U.S).

Swimming and swarming assays

The swimming and swarming motility activities were carried out according to the method of Garita-Cambronero *et al.* (2016). In swimming and swarming assays, 1 µl overnight grown cultures of the *X. oryzae* AS29 strain (OD_{600} nm 0.4) with and without specific concentrations of each EOs (1000 ppm in 10% DMSO) were point inoculated at the mid of the swimming and swarming agar medium consisting of LB medium with 0.3% and 0.5% agar, respectively. The inoculated plates were then incubated at 28 ± 2 °C without disturbance for 24 to 48 h. The reduction in swimming and swarming motility was observed by determining the zone formation of the *X. oryzae* AS29.

Exopolysaccharides (EPS) production

The effect of various EOs on EPS production was also measured. The target *X. oryzae* AS29 strain was grown in the presence and absence of specific concentrations of each EOs (1000 ppm in 10 % DMSO) at 28 ± 2 °C for 24 h. Further, *X. oryzae* AS29 cells were centrifuged at 8,000 rpm for 120 min. After centrifugation, the pellets were washed with 100 ml of buffer solution [K_3PO_4 (10 mM; pH 7), NaCl (5 mM), $MgSO_4$ (2.5 mM)] and centrifuged at 12,000 rpm for 20 min. The EPS was precipitated by adding three volumes of ethanol and stored at −20 °C. The extracted EPS was determined spectrophotometrically at 490 nm following the method described by Leroy and De Vuyst (2016).

Protease and lipase activity assays

For the analysis of extracellular protease and lipase activities, *X. oryzae* AS29 culture was grown overnight in liquid LB medium and adjusted to 0.4 at OD_{600} using SDW, and incubated at 28 ± 2 ^{0}C for 1 to 2 days on LB agar plates containing 1% skimmed milk (Himedia, India) and 1% tween 20 (Sigma-Aldrich) (Singh *et al.* 2017). The culture was point inoculated at the mid of the agar plates and the zone of activity was measured.

Statistical analysis

All the experiments were carried out thrice with six replicates and the mean values were calculated accordingly. The significance differences among various treatments were analyzed using Tukey's multiple comparison test (one-way ANOVA; $P \leq 0.05$).

RESULTS AND DISCUSSION

Biofilm formation and colony forming units (CFU)

The anti-biofilm activity of various EOs against *X. oryzae* AS29 revealed reduction in biofilm formation of *X. oryzae* AS29 strain when treated with 1000 ppm concentration of various EOs. Calamous oil (25%), nar kachur oil (46%), galangal oil (58%), cajeput oil (56%), juniper berry oil (5 %), costus root oil (51%), Jatamansi oil (48%), angelica root oil (47 %) and kulanjan oil (55%) at 1000 ppm efficiently dislodged the biofilm formation in XooAs29 (Table 1

and 2). Since, biofilm serves as a pool for antibiotic resistance genes in addition to providing refuge to bacteria from external attack; biofilm inhibition is being looked upon as a potential drug target for managing notorious bacterial pathogens (Sanchez *et al.* 2016; Singh *et al.* 2017). Recently, Kazemian *et al.* (2016) reported anti-swarming and anti-biofilm formation activities of *Chamaemelum nobile* against opportunistic human pathogen *Pseudomonas aeruginosa.*

Table 2: Effect of essential oils (1000 ppm) on biofilm forming potential of *X. oryzae* AS29. Mean value is an average of three replicates with ± standard error (SE). Tukey's multiple comparison test at a level of 5% of probability ($P \leq 0.05$) was applied. The averages followed by the same letter do not differ statistically between themselves

Sl. No.	Name of oil	Code	Absorbance ($O.D_{590}$ nm)
1	Calamous oil	ST21	0.72 ± 0.02^{b}
2	Nar Kachur oil	ST22	0.52 ± 0.05^{bc}
3	Galangal oil	ST26	0.40 ± 0.05^{c}
4	Cajeput oil	ST29	0.42 ± 0.02^{c}
5	Juniper berry oil	ST31	0.44 ± 0.03^{c}
6	Costus root oil	ST32	0.47 ± 0.06^{c}
7	Jatamansi oil	ST33	0.50 ± 0.05^{c}
8	Angelica root oil	ST34	0.57 ± 0.07^{bc}
9	Kulanjan oil	ST36	0.43 ± 0.01^{c}
10	Control (Only Xoo)	Cont.	0.96 ± 0.05^{a}

Table 3: Effect of essential oils (1000 ppm) on colony forming units of *X. oryzae* AS29. Mean value is an average of three replicates with ± standard error (SE). Tukey's multiple comparison test at a level of 5% of probability ($P \leq 0.05$) was applied. The averages followed by the same letter do not differ statistically between themselves

Sl. No.	Name of oil	Code	CFU (600nm)
1	Calamous oil	ST21	0.41 ± 0.03^{c}
2	Nar Kachur oil	ST22	0.46 ± 0.02^{bc}
3	Galangal oil	ST26	0.56 ± 0.08^{abc}
4	Cajeput oil	ST29	0.51 ± 0.07^{abc}
5	Juniper berry oil	ST31	0.49 ± 0.06^{bc}
6	Costus root oil	ST32	0.69 ± 0.05^{a}
7	Jatamansi oil	ST33	0.41 ± 0.01^{c}
8	Angelica root oil	ST34	0.56 ± 0.05^{abc}
9	Kulanjan oil	ST36	0.64 ± 0.08^{ab}
10	Control (Only Xoo)	Cont.	0.51 ± 0.02^{abc}

Since, EOs encompass an extensive range of antimicrobial phytochemicals showing potential activity against targets embedded in bacterial membrane and cytoplasm, the scope seems to be very promising. In line with the above reports, recently two volatile compounds namely carvacrol and eugenol were found to inhibit biofilm formation and quorum sensing controlled traits in Gram negative bacteria (Devi *et al.* 2010; Burt *et al.* 2014). Likewise, Lagonenko *et al.* (2013) reported that salicylic acid reduced the motility behaviour, biofilm and acyl homoserine lactone production in *Pectobacterium carotovorum* and *Pseudomonas syringae*. The effect of specific concentration of EOs on *X. oryzae* AS29 strain growth was evaluated by determining CFU at 600 nm (Table 3). The *X. oryzae* AS29 growth in the presence of maximum EOs (1000 ppm) did not affect the growth much significantly except for calamous, nar kachur, and jatamansi oils which showed significant reduction in bacterial growth at 1000 ppm.

Swimming and swarming assays

The anti-motility activity of various EOs was further determined for its ability to hinder swimming and swarming motility in *X. oryzae* AS29 strain. The results obtained clearly demonstrated the effect of EOs at 1000 ppm in inhibiting the motility of *X. oryzae* AS29 as shown in Table 4 and 5. For initiating infection, a bacterial pathogen must migrate from the epiphytic surface to the inside tissue of the infecting plant using motility and chemotaxis machinery (Pfeilmeier *et al.* 2016). In a study conducted by Antúnez-Lamas *et al.* (2016) showed that *Dickeya dadantii* 3937 mutanized in genes engaged in the chemotactic signal transduction system and flagellar motor showed reduced virulence and pathogenicity. In the present investigation, maximum reduction in swimming motility was observed in *X. oryzae* AS29 grown in the presence of calamous and cajeput oil (58%) whereas highest reduction in swarming motility was shown by Jatamansi (37%), costus root oil and kulanjan oil (33%) as compared to control XooAS29 grown in the absence of EOs (Table 4 and 5).

EPS production

Among the numerous strategies adopted by phytopathogenic bacteria to infect the host plant,

exoploysaccharides is reported to play a crucial role (Pfeilmeier *et al.* 2016). EPS is usually secreted outside bacterial cells in addition to forming protective layer around them. The importance of EPS in pathogenicity of many pathogenic bacteria is well documented (Melotto and Kunkel, 2013; Yu *et al.* 1999). EOs exhibited an inhibitory activity in reducing the EPS production of XooAS29. At the specific concentration of 1000 ppm, maximum reduction in the EPS production was observed in calamous (39%) and jatamansi oil (32%), followed by nar Kachur oil (29 %) over the control XooAS29 (Table 6). Thus, by targeting the non-essential pathways of the bacterium it is expected that the pathogenicity can be reduced to some extent by using the above oils.

Table 4: Effect of essential oils (1000 ppm) on swarming potential of *X. oryzae* AS29. Mean value is an average of three replicates with ± standard error (SE). Tukey's multiple comparison test at a level of 5% of probability ($P \leq 0.05$) was applied. The averages followed by the same letter do not differ statistically between themselves

Sl. No.	Name of oil	Code	Day 1	Day 2
1	Calamous oil	ST21	22 ± 1.73^{cd}	38 ± 2.30^{d}
2	Nar Kachur oil	ST22	25 ± 1.73^{bcd}	42 ± 1.15^{cd}
3	Galangal oil	ST26	32 ± 1.15^{abc}	60 ± 1.73^{b}
4	Cajeput oil	ST29	19 ± 1.73^{d}	38 ± 1.15^{d}
5	Juniper berry oil	ST31	30 ± 1.73^{abc}	60 ± 0.57^{b}
6	Costus root oil	ST32	38 ± 1.15^{a}	90 ± 2.30^{a}
7	Jatamansi oil	ST33	31 ± 1.15^{abc}	60 ± 1.15^{b}
8	Angelica root oil	ST34	32 ± 1.73^{abc}	60 ± 1.73^{b}
9	Kulanjan oil	ST36	33 ± 1.15^{ab}	50 ± 0.57^{bc}
10	Control (Only Xoo)	Cont.	40 ± 1.33^{a}	90 ± 1.15^{a}

Table 5: Effect of essential oils (1000 ppm) on swarming potential of *X. oryzae* AS29. Mean value is an average of three replicates with ± standard error (SE). Tukey's multiple comparison test at a level of 5% of probability ($P \leq 0.05$) was applied. The averages followed by the same letter do not differ statistically between themselves

Sl. No.	Name of oil	Code	Day 1	Day 2
1	Calamous oil	ST21	13 ± 1.73^{bc}	24 ± 1.73^{abc}
2	Nar Kachur oil	ST22	16 ± 2.30^{abc}	25 ± 1.15^{abc}
3	Galangal oil	ST26	17 ± 1.15^{ab}	28 ± 1.73^{ab}
4	Cajeput oil	ST29	14 ± 1.15^{bc}	25 ± 0.57^{abc}
5	Juniper berry oil	ST31	13 ± 1.73^{bc}	24 ± 1.73^{abc}
6	Costus root oil	ST32	11 ± 1.15^{bc}	20 ± 1.15^{c}
7	Jatamansi oil	ST33	9 ± 1.73^{c}	19 ± 1.73^{c}
8	Angelica root oil	ST34	10 ± 0.57^{bc}	21 ± 1.15^{bc}
9	Kulanjan oil	ST36	11 ± 1.73^{bc}	20 ± 1.73^{c}
10	Control (Only Xoo)	Cont.	22 ± 1.15^{a}	30 ± 2.30^{a}

Table 6: Effect of essential oils (1000 ppm) on exopolysaccharide production (EPS) of *X. oryzae* AS29. Mean value is an average of three replicates with ± standard error (SE). Tukey's multiple comparison test at a level of 5% of probability ($P \leq 0.05$) was applied. The averages followed by the same letter do not differ statistically between themselves

Sl. No.	Name of oil	Code	EPS production
1	Calamous oil	ST21	0.17 ± 0.03^{c}
2	Nar Kachur oil	ST22	0.19 ± 0.02^{bc}
3	Galangal oil	ST26	0.23 ± 0.08^{abc}
4	Cajeput oil	ST29	0.21 ± 0.07^{abc}
5	Juniper berry oil	ST31	0.20 ± 0.06^{bc}
6	Costus root oil	ST32	0.21 ± 0.05^{abc}
7	Jatamansi oil	ST33	0.17 ± 0.01^{c}
8	Angelica root oil	ST34	0.23 ± 0.05^{abc}
9	Kulanjan oil	ST36	0.26 ± 0.08^{ab}
10	Control (Only Xoo)	Cont.	0.28 ± 0.02^{a}

Protease and lipase activity assays

T2SS system is used by many pathogenic and non-pathogenic bacteria to insert the proteins into the extracellular surrounding of the host cells to suppress the basal defence reaction (Johnson *et al.* 2006). Among the major contributors of virulence; cell wall-degrading enzymes play an important role in setting off the disease symptoms (Benali *et al.* 2014).

In a study conducted by Aparna *et al.* (2009), lipA gene encoding lipase enzyme was needed for the complete virulence of *X. oryzae* pv. *oryzae*. Likewise, in *X. oryzae* many other enzymes like xylanases, proteases, cellulases etc. have been reported to be an important factor for bacterium's pathogenicity (Rajeshwari *et al.* 2005; Sun *et al.* 2005; Furutani *et al.* 2009). In the present study, clear zone formation representing positive activity was observed in all the treatments with respect to only control *X. oryzae* AS29.

However, the reduction was most prominent in calamous and jatamansi oil in case of protease while in case of lipase calamous and costus showed the best results (Fig. 1 and 2).

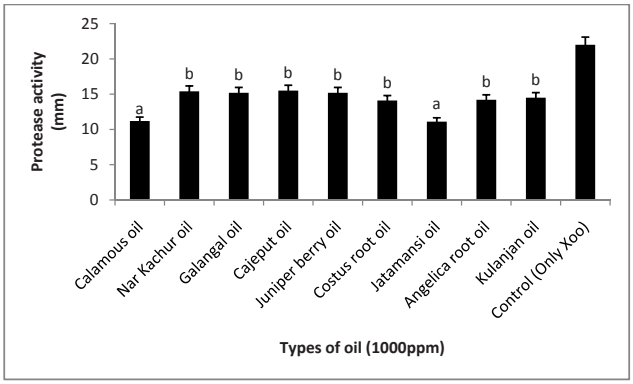

Fig. 1: Effect of essential oils (1000 ppm) on protease activity of X. oryzae AS29. Results are expressed as means of three replicates, and error bars indicate standard errors of the means. Different letters indicate significant differences among treatment as determined by Tukey's multiple comparison test at $P \leq 0.05$

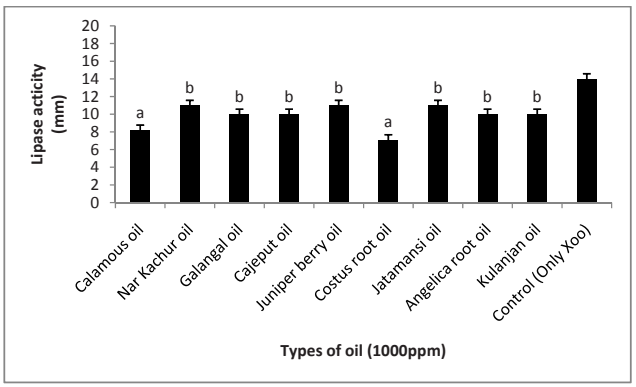

Fig. 2: Effect of oils (1000 ppm) on lipase activity of X. oryzae AS29. Results are expressed as means of three replicates, and error bars indicate standard errors of the means. Different letters indicate significant differences among treatment as determined by Tukey's multiple comparison test at $P \leq 0.05$

CONCLUSION

From the present study it can be concluded that few essential oils namely calamous, costus and jatamansi oil were in general overall effective in curbing down the virulence potential of phytopathogenic bacterium, *X. oryzae*. Since, plant and plant products are considerably safe, such oils in future can be used for the development of crop protectants against bacterial diseases. However, additional studies pertaining to its affect on non-target organisms needs to be done before taking them to the field conditions.

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