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Oscilene-e, an ethanolic extract producted from *Ocimum sanctum* L. leaves as biofungitoxicant in the management strategy of rice blast disease

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Abstract Oscilene-e, a formulated product was developed by combining ethanolic extract (EE) of *Ocimum sanctum* L. with a coded surfactant, A+ (FA) and bioassayed under *in vitro* condition against rice blast causing fungus (*Pyricularia grisea* Sacc.). Fungitoxic patterns such as complete inhibition, granulation in cytoplasm, reduction and granulation in germ tube length in conidial germination were exhibited in treatments in combination with FA and EE at concentrations from 10% to 0.0001%. Mycelial growth was completely inhibited at 0.1 percent concentration of the product, Oscilene-e. This formulated product retained its fungitoxicity in conidial germination distortion even after storage period of 24 months. In a separate test conducted under *in vivo* i.e. both in green house and under field conditions, it was found to be effective in reducing the foliar blast of rice crop and also in the reduction of the disease as observed comparable with the standard fungicide carbendazim.

Key words: surfactant; *Pyricularia grisea* Sacc., storage period; fungitoxic patterns; field condition

Introduction

Rice blast is a worldwide problem in rice and is dangerous because of its yield loss potential ranging upto 100% under favorable conditions (Yashida and Parao, 1976; Ou, 1985; Luo *et al.*, 1998; Netam *et al.*, 2011). Though disease control depends primarily on the application of synthetic chemicals, their overuse may result in undesirable residue left in food, water and environment, toxicity to human and animals as well as contamination of soils that may lead to development of crop pest population that are resistant to treatment with agrochemicals (Pretorius and Watt, 2011; Suwan *et al.*, 2012).

Concern over the excessful use of pesticide led researchers to select alternative methods that are environment-friendly and also relatively

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inexpensive compared with chemical pesticides (Choi *et al*., 2004; Tewari and Patra, 2006; Netam *et al*., 2011). Plant preparations such as aqueous extract, steamed aqueous extract, ethanolic extract and essential oil of *Ocimum sanctum* and *Aegle marmelos* have been successfully tried in laboratory at Central Rice Research Institute, Cuttack and were found to be effective against Phytopatogenic fungi viz. *Pyricularia grisea, Rhizoctonia solani, Aspergillus niger* and *Aspergillus flavus* (Tewari, 1998). The present work aimed to develop a formulation of *Ocimum sanctum* ethanolic extract thus enhancing its efficacy to be used as botanical fungicide.

Materials and methods

Preparation of Ethanolic Extract (EE)

Fresh leaves from *O. sanctum* weighing 5kg were collected, washed thrice in distilled water, air-dried in shade followed by oven drying for two hours at 45± 2 ℃ and powdered to get 420 gm dried powder. The powder was mixed in ethanol through Soxhlet apparatus (1:5w/v) following the method of Tewari (2008) the excess ethanol was evaporated. The extract recovered in syrupy form weighed 50 gm and was treated as mother extract which was successively diluted from 100%, 10%, 1%, 0.1%, 0.01%, and 0.001% to 0.0001% and utilized for further studies.

Preparation of formulated product

The formulating agent (FA), a surfactant coded A+ was similarly successively diluted from 100% ,10%, 1%, 0.1%, 0.01%, 0.001% to 0.0001% and each of these dilutions were combined with serially diluted EE (1:1v/v) and treated as Oscext-e which was subsequently used during the course of the investigation.

Isolation and maintenance of P. grisea

Actively growing fresh spindle-shaped leaf lesions of rice blast having brown margins and ashy grey centers were collected from a susceptible variety HR-12 , cut into small pieces, surface sterilized in 0.1% sodium hypochlorite solution for 30 seconds, washed thoroughly with sterilized distilled water thrice and dried on sterilized blotting paper before transferring aseptically into previously prepared Oat meal agar (OMA) medium [Oat meal-30g; Agar-Agar-20g; Biotin and Thiamine in traces; Distilled water-1L; Padmanabhan *et al.*, (1967)] in petri plates. The *P. grisea* isolate obtained was confirmed through

Koch's postulate. The isolate was further purified by single spore isolation and maintained on OMA slants. These slants were incubated for seven days at 24^0 C and stored at 4^0 C for further studies.

Bioassay test

Conidial germination test

Aliquots of 0.1 ml from each concentration viz., 10%, 1%, 0.1%, 0.01%, 0.001%, and 0.0001% of Oscilene-e, of the formulated product was pipetted out onto cavity slides separately and evaporated to dryness. Conidial suspension of 7day old pure culture of the test pathogen *P. grisea with* 30-35 conidia per microscopic field (Nene and Thapliyal, 1979) measuring 1.26 mm ^{2} were placed separately on each glass slide with equal quantity and incubated in moist chamber at 24^0C for 24hours. Observations on conidial germination (%) and the patterns of fungitoxicity were recorded using Olympus BX51 microscope at 10X magnification after 24 hours of incubation. Appropriate controls were maintained keeping three replications in each case and the experiment was repeated thrice. Data on germination was transformed to angular value and statistically analyzed.

Poisoned food technique

Oscilene-e was mixed with melted OMA media separately so as to get the final concentration of 1%, 0.1%, 0.01% and 0.001%. The extract mixed with medium was poured aseptically into petri plates and left for 5 days to allow ethanol to evaporate. Actively growing mycelia of *P. grisea* was cut with a sterile cork-borer (0.5 cm) and inoculated separately in the center of each petri plate aseptically. All plates were incubated at 28 ± 2^oC for seven days. Appropriate controls were maintained keeping three replications in each case and the experiment was repeated thrice. The mycelial growth (cm) was observed, recorded and computed using $3.14 \times r^2$ methods (Tewari and Shukla, 1990). No mycelial growth was accorded numerical value of 0.5 cm for the purposes of statistical analysis.

Shelf-life effect

To assess the fungitoxic effect of the formulated product under storage condition, Oscilene-e, FA and EE (100%) were stored at room temperature for 6, 12, 18 and 24 months in a clean, sterilized glass vial with air tight stopper. The product was then bioassayed separately against *P. grisea* conidial

germination at 1%, 0.1%, 0.01%, 0.001% and 0.0001% concentrations in the same way as previously done. Appropriate control was maintained keeping three replications in each case and the experiment was repeated thrice. Observations on conidial germination (%) and the pattern of fungitoxicity were recorded after 24 hours of incubation. Data on germination was transformed to angular value and statistically analyzed.

Dose response relationships studies under in vivo condition

Green house experiment

Healthy seeds of a blast susceptible rice variety HR12 were sown in 19cm diameter earthen pots filled with 3 kg sterilized soil mixed with compost with a ratio of 15:1. Pots were watered twice daily with tap water and ammonium sulphate was applied 20days after sowing at the rate of 1g/pot to accelerate disease development. Conidial suspension from 7-day old culture of *P. grisea* containing approximately 1,750 conidia per ml of sterilized distilled water was sprayed and inoculated into twenty five days old seedlings. EE, formulated agent (coded A+) and Oscilene-e were diluted to 1, 0.1 and 0.01% concentrations in aqueous suspension. These were sprayed thrice each at weekly interval separately on twenty-seven-day old seedlings showing initial blast symptoms. Standard fungicide carbendazim at 0.1% and sterilized distilled water were sprayed in the same way to serve as standard check and control respectively. The experiment was repeated thrice keeping three replications in each treatment. Observations on disease score (0-9 scale) was recorded on the fifth day of the last spraying. Data obtained were statistically analyzed.

Field experiment

Seeds of blast susceptible rice cultivar HR-12 were sown in lines on raised seed beds. Twenty five days old seedlings were transplanted in a randomized block design at two seedlings per hill in a 7x2.5 m plots with a spacing of 15 x 15 cm between hills and rows. Gap filling was done 7 days after transplanting. A gap of 1 m was left all around between plots. The plots were fertilized with N_{120}, P_{60} and K_{60} /ha as a basal dose. EE, FA (coded A+) and Oscilene-e (at 0.1% for spraying) was prepared. The extract was sprayed at weekly intervals three times beginning from initial symptom development of blast i.e. after 15^{th} day of transplanting. Standard fungicide carbendazim at 0.1% and sterilized distilled water were sprayed in the same way to serve as standard check and control respectively. All sprayings were carried out during morning

hours to avoid the scorching heat of the sun. Three replications were maintained for each treatments and the experiment was repeated thrice during the wet seasons of 2008-2010. The leaf area damage on the top three leaves barring flag leaves in three tillers per plant was recorded 7 days after last spraying and was expressed in percentage on five plants in each plot randomly selected leaving border line all around. The data gatered were statistically analyzed.

Statistical analysis

The data on conidial germination, mycelial growth, disease score and grain yield of FA and botanical have been taken as individual treatment and was statistically analysed after transforming the data to angular values using Cropstat 7.2 developed by IRRI. There was only one Critical Difference (CD) provided to compare between treatment means for all FA and botanical extract. The treatment means have been provided in a tabular form for a better and quick comparison.

Results

Conidial germination test

The product Oscilene-e, (FA+EE) treatments exhibited complete inhibition of the conidial germination up to 0.1% in FA with all combinations of EE and 0.01% of FA with 0.01 % EE. The least conidial germination (4% ± 1.62) was observed with combination of 0.001% EE with 0.01 % FA. Fungitoxic patterns ranged from granulation in cytoplasm of conidia to reduction and granulation in germ tube length at concentrations of 10% to 0.0001% of EE in combination with different concentrations of FA (Fig. 1). Control, FA and EE registered no conidial germination up to 0.1% respectively. Another control (sterilized double distilled water) registered a maximum conidial germination (98%) and was found to be significantly more [98 % (81.9) ± 1.62] than the product, Oscilene-e at 0.0001% of FA and 0.01% of EE (Table 1).

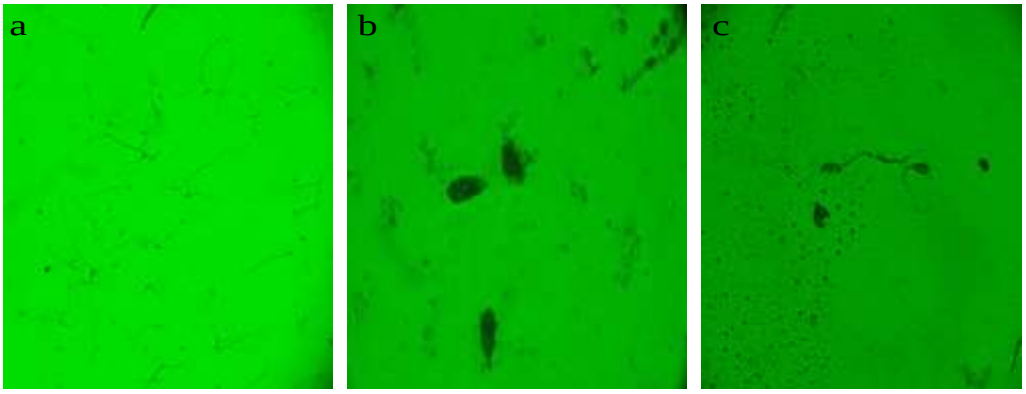

Fig. 1. Fungitoxic patterns in *P. grisea* .a= Normal germination. b= ungerminated conidia with granulated cytoplasm. c = granulation in germ tube and conidia.

Table 1. Fungitoxicity of Oscilene-e against *P. grisea* conidial germination (%)

Treatment	**CONCENTRATION (%)**						
(FA/EE)	10%	1%	0.1%	0.01%	0.001%	0.0001%	Control (FA)
10%	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)
1%	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)
0.1%	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	2 (8.13)	2 (8.13)	2 (8.13)
0.01	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	4[2,7] (11.54)	6[2,7] (14.18)	5[2,7] (12.92)
0.001%	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	30[1,2] (33.21)	40[1,7] (39.23)	95 (77.08)	50[2,7] (45.00)
0.0001%	2[1] (8.13)	2[1] (8.13)	85[1,7] (67.21)	90 (71.56)	98 (81.87)	98 (81.87)	98 (81.87)
Control(EE)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	40[1,2,6] (39.2)	80[1,6,10] (63.44)	98 (81.8)	-
Control	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87)	-

C.D. at P = 0.05 = 1.62 for interaction between individual treatments of EE, FA and formulated product. Data in parentheses represents angular values. Complete inhibition is represented as 2%. [1] cytoplasm granulated and/or aggregated in conidia. [2] reduced germ tube. [6]coiled/twisted germ tube. [7] germ tube granulated. [10] thin germ tube

Poisoned food technique

Oscilene-e (EE+FA) and EE alone produced complete inhibition up to 0.1% concentration (0.5cm) against *P. grisea.* Oscilene-e displayed significantly reduced mycelial growth (4.2 cm ± 0.16) at 0.001% concentration when compared with EE or FA, A+ tested alone (Table 2).

Table 1. Fungitoxicity of Oscilene-e against *P. grisea* mycelial growth

Treatment (EE/FA)	Concentration (%)				
	1	0.1	0.01	0.001	Control
	Mycelial growth (cm/cm^2)				
EE	0.5 (0.2)	0.5 (0.2)	4.2 (13.8)	4.5(15.9)	4.5(15.9)
FA(A+)	0.5 (0.2)	3.3 (8.5)	4.2 (13.8)	4.5(15.9)	4.5(15.9)
Oscilene-e (EE+FA)	0.5 (0.2)	0.5 (0.2)	3.0 (7.1)	4.2(13.8)	4.5(15.9)

C.D. at P = 0.05 =0.16 for interaction between individual treatments of EE, FA and Oscilene-e. Data in parentheses represents area of the mycelial growth in cm^2 computed through 3.14x r^2 method. Complete inhibition is represented by 0.5 cm/0.2cm^2

Shelf-life effect

Oscilene-e, EE and FA at 0.1% concentration showed complete inhibition (2%) against test pathogen *P. grisea* even after twenty four months of storage period. Oscilene-e retained its fungitoxicity up to twenty four months of storage period in all treatments except in EE (at 0.001%) at eighteen months storage, where the germination percentage significantly increased from eighty percent in twelve months to eighty four percent ± 1.40. Deformities pattern viz., granulated cytoplasm of conidia, thin, reduced and granulated germ tube were recorded in 0.001% concentration of formulated product, EE and FA alone after twenty four months storage (Table 3).

Greenhouse Test

All the treatments independently reduced significantly the disease over the control (5.5-7.0 ± 1.5). In both dry and wet seasons, the product reduced the disease and found at be at par or the same as with standard fungicide carbendazim at 0.1% concentration but the disease percentage was significantly higher in FA (1.8 -3.5 ± 1.5) followed by EE treatment (1.6 -3.3 ± 1.5) in 0.01 percent in both seasons and years. Disease in control ranged from 5.5 to 7.0 ± 1.5 (Table 4).

Table 2. Fungitoxic performance of Oscilene-e on rice blast disease reduction (0-9 scale) under *in-vivo* conditions

Year	Product	Dry Season			Wet Season		
		Concentration (%)					
		1	0.1	0.01	1	0.1	0.01
2008	EE	-	-	-	0.1	1.2	3.0
	FA	-	-	-	0.1	1.6	3.2
	Oscilene-e	-	-	-	0.1	0.4	2.2
	Carbendazim	-	-	-	-	0.5	-
	Control	-	-	-	6.7	6.7	6.7
2009	EE	0.1	0.9	1.9	0.1	1.1	2.9
	FA	0.1	1.0	2.2	0.1	1.5	3.3
	Oscilene-e	0.1	0.5	1.4	0.1	0.7	1.9
	Carbendazim		0.6	-	-	0.6	-
	Control	5.8	5.8	5.8	6.5	6.5	6.5
2010	EE	0.1	0.7	1.6	0.1	1.3	3.3
	FA	0.1	0.9	1.8	0.1	1.9	3.5
	Oscilene-e	0.1	0.4	1.0	0.1	0.5	2.4
	Carbendazim	-	0.2	-	-	0.7	-
	Control	5.5	5.5	5.5	7.0	7.0	7.0

C.D. at P =0.05 = 1.5 for interaction between individual treatments of EE, FA and Oscilene-e. No infection is accorded the value 0.1 for the purpose of statistical analysis.

Field experiment

Effect of the formulated product, Oscilene-e, EE and FA (coded A+) sprayed at 0.1% concentration was evaluated for the control of rice blast in fields during wet seasons of 2008 to 2010. All the treatments significantly reduced foliar blast compared to control, (76 % to 80% ± 4.0). In all three years, the product Oscilene-e reduced the disease (6% and 7% ± 4.0) and was found at par with a standard fungicide carbendazim (6% and 7% ± 4.0) at 0.1% concentration but the disease percentage was significantly higher in FA (A+) (28 % - 33% ± 4.00). In the year 2008 and 2009 the highest yield was reported in Oscilene-e (2403 Kg/ ha - 2422 Kg/ ha ± 4.00) which was found to be comparable to Carbendazim in all the three years (2340 Kg/ha -2420 Kg/ha ± 4.00). Untreated check produced lowest yield ranging from 935 to 875 Kg/ha ± 4.00 (Table 5).

Table 5. Fungitoxic performance of Oscilene-e on rice blast disease reduction (%) and grain yield (Kg/ha) at 0.1% concentration under field conditions

Treatment	Year					
	2008		2009		2010	
	DS (%)	GY (Kg/ha)	DS (%)	GY (Kg/ha)	DS (%)	GY (Kg/ha)
Ethanolic extract	21.0 (27.28)	2280	22.0 (27.97)	2250	24.0 (29.33)	2185
Formulating agent (A+)	28.0 (31.95)	1850	31.0 (33.83)	1792	33.0 (35.06)	1729
Oscilene-e	6.0 (14.18)	2422	7.0 (15.34)	2403	7.0 (16.43)	2338
Carbendazim	6.0 (14.18)	2420	6.0 (14.18)	2400	7.0 (14.18)	2340
Untreated	76.0 (60.67)	935	78.0 (62.03)	905	80.0 (63.44)	875

C.D. at P =0.05 = 4.0 for interaction between individual treatments of EE, FA and Oscilene-e. Data in parantheses represents angular value. DS=disease score. GY=Grain yield.

Discussion

Researchers are now engaged in looking for alternative sources to synthetic fungicide due to the adverse effect of latter being encountered by the end users through reports more now than ever before. Botanical products are being considered since long time in the past as safer over synthetic fungicides coupled with additional advantage such as these have more than one active ingredient making it difficult for the targeted organism to develop resistance to them. Certain group of plants, those which are aromatic in nature are characteristically rich in compounds with antimicrobial properties. Many of these compounds are terpenes having fungicidal properties. Few studies have focused on mechanism by which plant extracts and their essential oil inhibit phytopathogenic fungi (Martinez, 2012). Tewari (1995) reported significant inhibition of both conidial germination and mycelial growth under *in-vitro* condition from *Ocimum sanctum* leaf extract. Fungitoxic patterns viz. septal dissolution, short and deformed germ tube in germinated conidia, fluffy, delayed/no sclerotial production in mycelial growth was reported against *P.grisea*, *Aspergillus niger* and *Rhizoctonia solani* (Tewari and Mishra, 1990; Tewari, 2008).

The rapid decomposition of unformulated plant products if not utilized fresh, hinders its application as crop protectant, therefore the development of an effective formulated botanical product becomes necessary (Upadhyaya *et al*., 2012). In this study, a new product Oscilene-e developed from *O. sanctum*

ethanolic extract was tested against economically important blast disease of rice. This formulated product has not only been found to inhibit the conidial germination and mycelial growth but also produced distortion patterns such as reduced and granulated germ tube and/ or granulation of conidial cytoplasm and reduced mycelial growth (Tables 1 and 2) compared to control that produced normal conidial germination and mycelial growth. Under *in vivo* conditions, effective reduction in foliar blast percentage and highest yield was recorded in case of Oscilene-e treatment that was found to be comparable to a standard synthetic fungicide carbendazim in green house and under field conditions (Tables 4 and 5).

The shelf- life of such value added formulated product retained its fungitoxicity effectively for a period of 24 months, in all the treatments except in EE wherein within 18 months of storage the efficacy of the product was found reduced but not in other treatments (Table 3). It is therefore established through this study that improved efficacy of the product Oscilene-e would enable the users to utilize the product readily at the time of need without compromising the advantageous fungitoxic strength of the product saving cost involved in repeated preparation of the readily decomposable unformulated product. Thus the formulated product developed and reported herewith possesses the potential to be deployed in blast disease management strategy.

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Table 3. Oscilene -e (EE +FA) shelf - life effect against *P. grisea* conidial germination

Concentration (%)	**Storage period (months)**														
	Fresh			6			12			18			24		
	Conidial germination (%)														
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
1	2^{1} (8.13)	2^{1} (8.13)	2^{1} (8.13)	2^{1} (8.13)	2^{1} (8.13)	2^{1} (8.13)	2^{1} (8.13)	2^{1} (8.13)	2^{1} (8.13)	2^{1} (8.13)	2^{1} (8.13)	2^{1} (8.13)	2^{1} (8.13)	2^{1} (8.13)	2^{1} (8.13)
0.1	2^{1} (8.13)	2^{1} (8.13)	2 (8.13)	2^{1} (8.13)	2^{1} (8.13)	2 (8.13)	2^{1} (8.13)	2^{1} (8.13)	2 (8.13)	2^{1} (8.13)	2^{1} (8.13)	2 (8.13)	2^{1} (8.13)	2^{1} (8.13)	2 (8.13)
0.01	2^{1} (8.13)	$40^{1,2,6}$ (39.23)	$5^{2,7}$ (12.92)	2^{1} (8.13)	$40^{1,2,6}$ (39.23)	$5^{2,7}$ (12.92)	2^{1} (8.13)	$40^{1,2,6}$ (39.23	$5^{2,7}$ (12.92	2^{1} (8.13)	$40^{1,2,6}$ (39.23)	$5^{2,7}$ (12.92)	2^{1} (8.13)	$47^{1,2,6}$ (43.28)	$5^{2,7}$ (12.92)
0.001	$40^{1,7}$ (39.23)	$80^{1,6,10}$ (63.44)	$50^{2,7}$ (45.00)	$40^{1,7}$ (39.23)	$80^{1,6,10}$ (63.44)	$50^{2,7}$ (45.00)	$40^{1,7}$ (39.23)	$80^{1,6,10}$ (63.44	$50^{2,7}$ (45.00	$40^{1,7}$ (39.23	$84^{1,6,10}$ (66.42)	$50^{2,7}$ (45.00)	$40^{1,7}$ (39.23)	$90^{1,10}$ (71.56)	$50^{2,7}$ (45.00)
0.0001	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87	98 (81.87	98 (81.87	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87)
Control	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87	98 (81.87	98 (81.87	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87)	98 (81.87)

C.D. at P=0.05 = 1.40 for interaction between individual treatments of EE, FA and Oscilene-e. Data in parentheses represents angular values. [I] Oscilene-e. [II] EE. [III] FA ' A+'.Complete inhibition is accorded value 2% for statistical analysis . [1] cytoplasm granulated and /or aggregated in conidial cell. [2] reduced germ tube. [6] coiled germ tube. [7] granulated germ tube. [10] thin germ tube