Efficacy of an organic formulated product developed from *Ocimum sanctum* L. essential oil as plant protectant especially in Rice blast disease management

Supreet Upadhyaya, Anand Prakash, S.N. Tewari

Abstract— The residual effect of the organically derived product is relatively shorter than synthetic products such as fungicides like carbendazim etc. when applied extraneously on the surface of host plant. Hence *Ocimum sanctum* essential oil was combined with a formulating agent (coded A+) and named Oscilene-eo, bioassayed under *in-vitro* condition against *Pyricularia grisea* Sacc. causing blast disease of rice. Fungitoxic patterns such as complete inhibition, granulation in cytoplasm, branched and granulated germ tube registered in conidial germination coupled with complete inhibition of mycelial growth at 0.1 percent through various experiments confirms the fungitoxic strength of the formulated product. Oscilene-eo retained its fungitoxicity till 24 months storage period in all treatments. In a separate test of the product in green house and under field condition, it was not only found effectively reducing the foliar blast of rice crop but also found at par with a standard fungicide Carbendazim (Bavistin 50% w.p.) at 0.1% concentration.

Index Terms— *Ocimum sanctum*, *Pyricularia grisea* Sacc., Blast, Oscilene-eo, fungitoxic patterns, storage period, field condition
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1 INTRODUCTION

IT It has been estimated that half of the world's population subsists wholly or partially on rice. Ninety percent of the world's crop is grown and consumed in Asia[1] This crop is attacked by number of diseases of which blast incited by *Pyricularia grisea* Sacc is considered one of the most destructive disease due to its wide distribution causing severe losses in grain yield ranging from 50-100% [2] . Synthetic fungicide though, are primary means in controlling the disease but resistance development continues to be a challenging issue. Whereas, essential-oil-based formulations owing to the complex mixtures of constituents in it that characterizes many of these oils that makes the process of resistance development slow in phytopathogenic fungi[3]. The essential oil formulations are many times less toxic than synthetic chemicals, thus being exempted from toxicity list of EPA in United states [4]. Essential oil constituents are actually beneficial to human health when acquired through diet, but these get degraded (non persistent) easily in soil, freshwater and under aerobic conditions at certain temperature [5]. Hence their use can minimize the consequences in terms of ecological damage and serious negative impact on human health due to indiscriminate and high application frequency of synthetic fungicides [6, 7, 8, 9]. This encourages to conduct studies on development of the bio-based product from the natural sources to inhibit the phytopathogenic fungi [10, 11]. The investigation reported as hereunder are on developing an appropriate formulation for enhancing the efficacy and shelf life of such botanical products so as to prevent the residual toxic effect compare to when synthetic chemicals are used for control of disease.

2 MATERIAL AND METHODS

2.1 Preparation of Essential oil (EO)

Fresh leaves from *O.sanctum* weighing 1kg were washed thoroughly, loaded in Clevenger's apparatus and Sterilized double distilled water is added to it (1:1w/v). Essential oil (5 ml) was collected and moisture from the oil was separated utilizing differential freezing point principle [12] .The remaining moisture was removed by addition of Sodium Sulphate and pure essential oil was decanted in a clean sterilized glass vial. The Essential oil was diluted from 100% to, 10%, 1%, 0.1%, 0.01%, 0.001% and 0.0001% and utilized for further studies..

2.2 Preparation of Formulated product (Oscilene-eo)

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

2.3 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 it to previously

prepared Oat meal agar (OMA) medium (Oat meal-30g; Agar-Agar-20g; Biotin and Thiamine in traces; Distilled water-1L; Padmanabhan et al.[13]) aseptically in petriplate. The *P.grisea* isolate thus obtained was confirmed through Koch's postulate, 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.

2.4 Bioassay test

2.4.1. Conidial germination test

Aliquots, 0.1 ml from each concentrations viz., 10%, 1%, 0.1%, 0.01%, 0.001%, and 0.0001% of Oscext-eo, the formulated product was pipetted out on to 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 (1.26 mm^2) were placed separately on each glass slide with equal quantity and incubated in moist chamber at 24^0C for 24hours (Nene and Thapliyal[14]). 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.

2.4.2. Poisoned food technique

Oscilene-eo was combined with melted OMA media separately so as to get the final concentration of 1%, 0.1% , 0.01% and 0.001%. The extract mixed media was poured into the petriplates aseptically and inoculated after 4 days allowing ethanol to be evaporated fom the media meanwhile, as also in control plate . Removed contaminated plates. Actively growing mycelia of *P. grisea* was cut with a sterile cork-borer and inoculated separately in the center of each such petriplates aseptically. All such plates were incubated at 28±2°C 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 and recorded when it grew to periphery in control petriplates and was computed through $3.14 \times r^2$ methods [15]. No mycelial growth was accorded numerical value 0.5 cm, for the purpose of statistical analysis.

2.5 Shelf-life studies

Shelf-life effect of Oscilene-eo, FA and EO (100%) stored at room temperature for 6,12, 18 and 24 months in a cleaned, 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 stated earlier in text. 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.

2.6 Dose-response relationship studies under in-vivo conditions

2.6.1. 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 in the ratio 15:1. Pots were watered twice daily with tap water and ammonium sulphate was applied after 20days of sowing @1g/pot to accelerate the disease development. Conidial suspension from 7-day old culture of P.grisea (containing 30-35 conidia per microscopic field under low power magnification) prepared as described earlier and sprays inoculated on twenty-five-day old seedlings. EO, formulating agent (A+ coded) and Oscilene-eo, 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 @ 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 [16]. Data obtained were statistically analyzed.

2.6.2. 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 @ 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 plot to plot. The plots were fertilized with N120, P60 and K60 /ha as a basal dose. EO, FA (A+ coded) and Oscilene-eo (@ 0.1% for spraying) was prepared as stated earlier. The extract was sprayed at weekly intervals three times beginning from initial symptom development of blast i.e. after 15th day of transplanting. Standard fungicide carbendazim @ 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 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 damaged on the top three leaves barring flag leaves in three tillers per plant was recorded 7 days after last spraying in percentage on five plants in each plot

- *Supreet Upadhyaya is Research Scholar in Central Rice Research Institute, Odisha, India, Mobile no. +919040566162. E-mail: supreet.upadhyay29@gmail.com,*
- *Anand Prakash, is currently Principal scientist in Central Rice Research Institute, Odisha, India.*
- *S.N. Tewari is currently Principal scientist in Central Rice Research Institute, Odisha, India E-mail: supali29@gmail.com , sntewari52@gmail.com*

randomly leaving the border line all around. Data were statistically analyzed.

2.7 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 is only one CD provided to compare between the treatment means for all FA and botanical. The treatment mean values have been provided in a tabular form for a better and quick comparison and also to economize space in publication of the paper.

3 RESULTS

3.1 Conidial germination test

The product Oscilene-eo, (EO+A+) treatments exhibited complete inhibition of the conidial germination at 0.01% in FA (A+) with all combinations of EO and 0.0001% of A+ with 0.01 % EO . The least conidial germination (25% ± 1.58) was observed with combination of 0.001% EO and 0.001 % A+. Fungitoxic patterns ranged from granulation in cytoplasm of conidia to branching and granulation in germ tube length at concentrations 10% to 0.0001% of EO in combination with different concentrations of A+ (Fig. 1). Control FA (A+) and EO registered no conidial germination at 0.1% and 0.01% respectively. Another control (ethanol) registered a maximum of 98% conidial germination and was found significantly more [98 % (81.9) ± 1.58] than the product, Oscilene-eo at 0.0001% of FA and 0.0001 % of EO (Table 1)

3.2 Poisoned food technique

Oscilene-eo (EO+A+) and EO alone produced complete inhibition upto 0.1% concentration (0.5cm) against *P. grisea* . Oscilene-eo displayed significantly reduced mycelial growth (3.8cm ± 0.30) at 0.001% concentration when compared with either EO or FA, A+ tested alone (Table-2).

3.3 Shelf-life effect

Oscilene-eo and FA, A+ at 0.01% and 0.1% concentration respectively showed complete inhibition (2%) against test pathogen P. grisea till twenty four months storage period. Oscilene-eo retained its fungitoxicity up to twenty four months storage period in all treatments whereas EO (at 0.001%) in eighteen months storage, registered germination percent, significantly increased from thirty three percent in twelve months to thirty seven percent ± 3.0 . Deformities patterns viz., granulated cytoplasm of conidia, thin, branched and granulated germ tube were recorded up to 0.0001% concentration of formulated product till twenty four months storage period (Table 3).

TABLE 1
FUNGITOXICITY OF OSCILENE-EO AGAINST *P. GRISEA* CONIDIAL GERMINATION (%)

Treatment (FA/EO)	Concentration (%)						
	10	1	0.1	0.01	0.001	0.0001	Control (FA)
	Conidial germination (%)						
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[1] (8.13)	2[1] (8.13)	2 (8.13)
0.01	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	5 [2,7] (12.92)
0.001%	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	25 [2,7] (30.00)	82[3,7] (64.90)	50[2,7] (45.00)
0.0001%	2 (8.13)	2 (8.13)	2 (8.13)	2 (8.13)	80 [1,3,7] (63.44)	89 [6,10] (70.63)	97 (80.02)
Control (EO)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	2[1] (8.13)	30 [1,2] (33.21)	90[1,6,10] (71.56)	-
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 = 1.58 for interaction between individual treatments of EO, 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. [3] branched germ tube, [6]coiled/twisted germ tube. [7] granulated germ tube . [10] thin germ tube

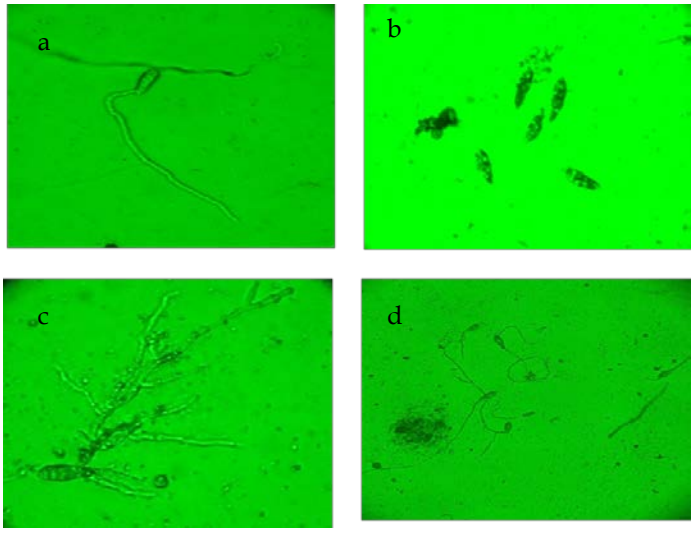

Fig.1. Fungitoxic patterns in *P. grisea*. [a] Normal germtube. [b] Ungerminated conidia. [c] Branched, granulated germtube. [d] Thin coiled germtube.

TABLE 2
FUNGITOXICITY OF OSCILENE -EO AGAINST *P.GRISEA* MYCELIAL GROWTH

Concentration (%)	Treatment		
	Oscilene-eo	EO	FA
	Mycelial growth (cm/cm²)		
1	0.5 (0.2)	0.5 (0.2)	0.5 (0.2)
0.1	0.5 (0.2)	0.5 (0.2)	3.3 (8.5)
0.01	2.0(3.14)	3.0 (7.1)	4.2 (13.8)
0.001	3.8 (11.34)	4.5 (15.9)	4.5 (15.9)
Control	4.5 (15.9)	4.5 (15.9)	4.5 (15.9)

C.D. at P = 0.05 = 0.30 for interaction between individual treatments of EO, FA and Oscilene-eo. Data in parentheses represents area of the mycelial growth in cm² computed through 3.14x r² method. Complete inhibition is represented by 0.5 cm/0.2cm² .

3.4 Green house test

All the treatments did significantly reduce the disease compared to control (6.5-7.0 ± 1.50). Independently in each year, Oscilene-eo reduced the disease and found at par with standard fungicide carbendazim (0.5- 0 .7 ± 1.50) in the test years 2008 and 2010, at 0.1% concentration but the disease score was significantly higher in FA (3.2 -3.5 ± 1.50) and EO treatment (2.7 -3.1 ± 1.50) in 0.01 percent in all the three years (Table 4).

3.5 Field experiment

All the treatments significantly reduced foliar blast compared to control, (76 % - 80% ± 2.50) over the years of experimentation. Oscilene-eo reduced the disease to 6% - 8 % ± 2.50 which was found significantly at par with a standard fungicide carbendazim (6%, 7% ± 2.50) at 0.1% concentration in both but the disease percentage was significantly higher in FA (A+) sprayed at corresponding concentration (28 % - 33% ± 2.50). Higher yield was reported in Oscilene- eo [2423 Kg ha^{-1} (2008), 2402 Kg ha^{-1} (2009) and 2341 Kg ha^{-1} (2010) ± 2.50] which was at par with Carbendazim (2400 Kg ha^{-1}, 2340 Kg ha^{-1} ± 2.50) in years, 2009 and 2010 . Untreated check produced lowest yield in the range of 875-935 Kg ha^{-1} ± 2.50, in all the three years which was significantly less than any other treatment (Table 5).

TABLE 4
FUNGITOXIC PERFORMANCE OF OSCILENE- EO AGAINST RICE BLAST DISEASE REDUCTION UNDER *IN-VIVO* CONDITIONS DURING WET SEASON

Treatment	Years								
	2008			2009			2010		
	Concentration (%)								
	1	0.1	0.01	1	0.1	0.01	1	0.1	0.01
	Disease score (0-9 scale)								
EO	0.1	1.0	2.7	0.1	1.0	3.0	0.1	1.1	3.1
FA	0.1	1.6	3.2	0.1	1.5	3.3	0.1	1.9	3.5
Oscilene-eo	0.1	0.4	1.6	0.1	0.5	1.3	0.1	0.6	1.9
Carbendazim	-	0.5	-	-	0.6	-	-	0.7	-
Control	6.7	6.7	6.7	6.5	6.5	6.5	7.0	7.0	7.0

C.D. at P =0.05 = 1.50 for interaction between individual treatments of EO, FA and Oscilene-eo. No infection is accorded the value 0.1 for the purpose of statistical analysis

TABLE 5

. Fungitoxic performance of Oscilene -eo on rice blast disease reduction (%) and grain yield (Kg ha^{-1}) at 0.1 % concentration under field condition

Treatment	Year					
	2008		2009		2010	
	DS (%)	GY (Kg ha^{-1})	DS (%)	GY (Kgha^{-1})	DS (%)	GY (Kg ha^{-1})
Essential oil	12.0 (20.27)	2330	15.0 (22.79)	2310	18.0 (25.10)	2260
Formulating agent (A+)	28.0 (31.95)	1850	31.0 (33.83)	1792	33.0 (35.06)	1729
Oscilene-eo	6.0 (14.18)	2423	7.0 (15.34)	2402	8.0 (16.43)	2341
Cabendazi-m	6.0 (14.18)	2420	6.0 (14.18)	2400	7.0 (15.34)	2340
Untreated	76.0 (60.67)	935	78.0 (62.03)	905	80. 0 (63.44)	875

C.D. at P =0.05 = 2.5 for interaction between individual treatments of EO, FA and Oscilene-eo. Data in parentheses represents transformed angular values. DS disease score, GY Grain yield

4 Discussion

Bioactive compounds viz. essential oils are synthesized by plants as secondary metabolites that possesses varied fungitoxic effect on the development of not only the mycelial growth of fungi and effect on sporulation rate but also inhibition in germination ranging from fungistatic effect to complete inhibition [17]. Plant species such as *Fluorensia* spp., *Opuntia* spp., *Piper longum*, *Tagetes patula*, exhibited fungicidal activity against phytopathogenic fungi *Alternaria* spp., *Rhizoctonia solani*, *Fusarium oxysporium*, *Pyricularia oryzae*, *Botrytis cineria* and many more [9, 18, 19, 20]. Formulations of plant extracts from *O.sanctum* and *Aegle marmelos* produces fungitoxic patterns such as disruption in cell membrane integrity, bursting of cell wall in conidial germination and reduction in mycelial growth and sporulation. Restricted and particularly discontinuous radial growth was reported with *P. grisea*, *Aspergillus niger* and *R. solani* pathogens (12, 21, 22).

Keeping in view the rapid decomposition of unformulated product [23] the present study was undertaken to develop the *Ocimum sanctum* essential oil based formulated product in Natural Plant Product Laboratory, Division of Crop Protection at Central Rice Research Institute, Cuttack. This product developed was named Oscilene-eo and and its fungitoxicity was bioassayed both under *in vitro* and *in vivo* conditions. Oscilene-eo inhibited the conidial germination and mycelial growth producing fungitoxic patterns ranging from granulation in conidial cytoplasm to branched, granulated germtube in the former and restricted growth compared to unformulated product in latter case. Under greenhouse and field condition the formulated product controlled disease effectively, and found at par with synthetic fungicide, Carbendazim (Bavistin 50% w. p.) treatment. Under field conditions, the formulated product, Oscilene-eo produced highest grain yield that was found at par with carbendazim in years 2009 and 2010 (Table 5).

The shelf- life of this value added formulated product retained its fungitoxicity effectively for a period of 24 months, in all the treatments except of course in EO in 18 months storage, where the efficacy of product was found reduced (Table 3). It is therefore established through the study that thus improved efficacy of Oscilene-eo would save the cost involved needed for repeated preparation of the unformulated product and enable the users to utilize the product readily at the time of need without compromising the advantageous fungitoxic strength of it. Thus the formulated product developed and reported herewith possess the potential to be deployed in blast disease management strategy.

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OSCILENE-EO (EO +FA) SHELF - LIFE EFFECT AGAINST *P.GRISEA* CONID-IAL GERMINATION

Concentration (%)	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)	2^{1} (8.13)	$5^{2,7}$ (12.92)	2^{1} (8.13)	2^{1} (8.13)	$5^{2,7}$ (12.92)	2^{1} (8.13)	2^{1} (8.13)	$5^{2,7}$ (12.92)	2^{1} (8.13)	$10^{1,2}$ (18.44)	$5^{2,7}$ (12.92)	2^{1} (8.13)	$20^{1,2}$ (26.56)	$5^{2,7}$ (12.92)
0.001	$25^{2,7}$ (30.00)	$30^{1,2}$ (33.21)	$50^{2,7}$ (45.00)	$25^{2,7}$ (30.00)	$30^{1,2}$ (33.21)	$50^{2,7}$ (45.00)	$25^{2,7}$ (30.00)	$33^{1,2}$ (35.06)	$50^{2,7}$ (45.00)	$25^{2,7}$ (30.00)	$37^{1,2}$ (37.47)	$50^{2,7}$ (45.00)	$25^{2,7}$ (30.00)	$45^{1,2}$ (42.13)	$50^{2,7}$ (45.00)
0.0001	$89^{6,10}$ (70.63)	$90^{1,6,10}$ (71.56)	98 (81.87)	$89^{6,10}$ (70.63)	$92^{1,6,10}$ (73.57)	98 (81.87)	$89^{6,10}$ (70.63)	$92^{1,6,10}$ (73.57)	98 (81.87)	$89^{6,10}$ (70.63)	98^{1} (81.87)	98 (81.87)	$89^{6,10}$ (70.63)	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)

Storage period (months)

C.D. at P=0.05 = 3.0 for interaction between individual treatments of EO, FA and Oscext-eo. Data in parentheses represents angular values. [I] Oscilene-eo. [II] EO. [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