J. Mycopathol, Res, ***51(1)*** : 107-112, 2013; (ISSN 0971-3719)

Effect of leaf extract and volatile oil of *Ocimum* on the growth of some selected phytopathogens-a novel approach for biocontrol of plant diseases

P.GIRI[1], P.D.GHOSH[2], AND S.K. MUKHERJEE[3]
[1]Dept. of Botany, Dum Dum Motijheel College, Dum Dum, Kolkata 700 000, West Bengal
[2 & 3]Department of Botany, Kalyani University, Kalyani, Nadia, West Bengal

Received : 20.01.2012 Accepted : 21.09.2012 Published : 29.04.2013

Ocimum is used profusely in Ayurvedic medicine and as a constituent of different herbal preparations. Medicinal importance of these species is correlated with their antimicrobial properties. In our present investigation the effect of leaf extract and volatile oil from all those species was tested against two pathogenic microorganisms such as *Pseudomonas* solanacearum and *Alternaria brassisicola*. It was observed that maximum growth inhibition of *P.* solanacearum occurs in presence of volatile oil as well as leaf extract of *Ocimum gratissimum*. On the other hand maximum inhibition in the growth of *Alternaria brassisicola* was recorded in the medium supplemented with the volatile oil and leaf extract of *Ocimum americanum*. The major constituents of the volatile oil and their concentration were determined through GC analyses and the data obtained were tried to correlate with their antimicrobial efficacy. It was observed that among the six species five species have the common constituent as eugenol in their essential oil which may be responsible for their common growth inhibitory effect on *P. solanacearum*. The degree of inhibition is also correlated with the percentage of eugenol content in the essential oil. As the *eugenol* content is maximum in *O. gratissimum* (47.48%) oil, the degree of inhibition of growth against the organism was highest. In *O. americanum* however, camphor, cineole in addition to eugenol suppose to be responsible for maximum inhibition of mycelial growth of *A. brassisicola*. The growth inhibitory effect of *O. kilimandscharicum* in absence of *eugenol* may be due to the interaction of other compounds in its oil. The similar growth inhibitory effects of the various solvent extracts of leaf on the test organism again corroborate with the fact that eugenol is the common inhibitory substance occurs in the volatile oil in different species of *Ocimum*. Being heterogeneous in nature of the constituents, apart from *eugenol*, other inhibitory substances may be present in oil which may be in collaboration or an independent manner may perform inhibitory action.

Therefore, there is a need of thorough phytochemical study of various species of *Ocimum* regarding its constituents that are growth inhibitory in nature. So, apart from herbal medicinal use, there is a distinct possibility to explore *Ocimum* as a potential biocontrol agent against phytopathogens. In such case dried leaves may be used to amend the soil to retard the survival of soil borne phytopathogens.

Key words : *Ocimum,* Leaf extract, Volatile oil, Biocontrol, Plant diseases

INTRODUCTION

In recent year there is a growing interest in the search of effective alternative methods for controlling plant pathogens since the conventional methods like applications of different chemicals for retardation of the growth of phytopathogens are not ecofriendly and being non degradable in nature rather they create environmental hazards. Moreover, phytopathogens sometimes develop resistance against such chemicals making such approach ineffective.

The development of plant based biocontrol agent is therefore a challenging area of research and

E-mail: giri_pranab@yahoo.in

need based ones considering their safety, affectivity, biodegradability and long lasting prcperty. Green plants in nature are the reservoir of wide variety of bioactive principles which could be exploited for development of effective formulations to control different phytopathogens (Evans 2002). Therefore, plant extracts are analyzed intensively in order to isolate novel bioactive compounds.

Ocimum is represented by six species (Banerjee, 1996) a herbaceous aromatic Labiate plant growing widely in different regions of West Bengal, and is used profusely in herbal medicine to cure various ailments of human beings. The antimicrobial activity of leaf extracts and essential oil of different species under this genus have been well documented but the report on its exploitation as biocontrol agent of phytopathogens in relation to develop effective bioactive formulations is scanty. In our present investigation efforts have been made to test the anti phyto pathogenic potentiality of all species of this plant growing in West Bengal with the view to prescribe its essential oil as a constituent of bioactive formulations. In this regard, two test organisms such as *Pseudomonas solanacearum* (= *Ralstonia solanacearum*) and *Alternaria brassisicola* have been selected to study their growth inhibition in presence leaf extract as well as essential oil of all the available six species. The former is a common solanaceous bacterial wilt causing pathogen whereas the later is responsible for causing black spot disease of crucifer. Attempts have also been made to correlate the constituents of essential oil of the species with their degree of inhibitory effect on the selected plant pathogens.

MATERIALS AND METHODS

Extraction of essential oil from leaves of different species of ocimum

Oil sample was extracted from fresh leaves following hydrodistillation method using cleavenger's apparatus and extraction was made with petroleum ether (b.p. 40^0 C -60^0 C). Extracts dried over by anhydrous Na_2So_4 (30 gm/l) and solvent distilled off under reduced pressure (Clevenger,1928).

Methods for preparation of leaf extract

10 gms of dry leaves of each plant species were washed 2-3 times with tap water and distilled water and then surface sterilized with 90% alcohol. Subsequently, the plant materials were grinded in a mortar pestle using 10 ml of different solvents separately. For experimental purpose ethanol, methanol, petroleum ether, acetone extracts were used. The macerates were kept at room temperature to evaporate the solvents. In the remaining residue, 10 ml of distilled water was added. Macerates were squeezed through double layered muslin cloth and filtered through filter paper. The aliquots were then centrifuged at 10,000 rpm for 20 min. The supernatant were filtered through Whatman No. 1 filter paper and then sterilized by passing through 0.2 µ disposable filters. The aqueous extracts of leaves of different species were prepared separately by grinding the leaves in 10 ml distilled water. After that the extraction was made following same procedure as mentioned earlier.

Evaluation of antibacterial effect using leaf extract

For evaluation of antimicrobial effect of plant extracts, microbial bioassay method was followed (Daswani and Bohra, 2002). Nutrient broth medium was prepared and distributed in several culture tubes, each with 10 ml of medium. The medium was sterilized by autoclaving. To each tube containing 10 ml of medium, 0.2 ml of 24 hrs old culture of test organism (grown in nutrient broth) and 1 ml of filter sterilized extracts were added. One blank control was prepared. Another control was prepared by adding 1 ml of sterilized distilled water with the tube containing 10 ml of medium plus 0.2 ml of inoculum. Three replicas were taken for each extract. These tubes were then incubated in BOD incubator with shaker for 24 hrs at 37^0C. The optical density of each set were measured at 540 nm and compared with the control.

Evaluation of antifungal activity of leaf extract

10 ml of culture medium (Czapek's Dox) was taken in a conical flask. The medium was sterilized. To the medium, 0.1 ml of fungal spore suspension and 1 ml of filter sterilized extract was added. One control set was prepared by adding 0.1 ml inoculum and 1 ml of sterilized distilled water. For each treatment three replicas were prepared. The control and treatment sets were incubated in shaking condition for 24 hrs at 32^0C in a BOD incubator. The mycelial mass was filtered and then dried in hot air oven. The difference in the dry weight from the control set was considered as growth inhibition.

Evaluation of antibacterial and antifungal activity of essential oil

Antibacterial and antifungal activity of the essential oil sample was determined by disc diffusion method. Fungal inoculum (0.1ml) was applied on the surface of Czapek's Dox agar (Diffco) plate and spread with the help of a sterile glass spreader. The CFU/ml of the inoculum (spore suspension) of the test fungus was adjusted $1x10^5$before spreading over solid agar plates. The antibacterial activity of essential oil was also studied following the same method using nutrient agar medium. Sterile disc of 8mm diameter were soaked in different essential oil sample and placed on the solid agar plate. The plates were incubated at 28°C for 48 hours. The diameter of the radial inhibition indicates the antimicrobial action of the essential oil sample. The MIC of the essential oil was also determined similarly and for that purpose various concentration of oil sample was prepared by dissolving it in dimethyl sulfoxide (DMSO) with Tween 80 (0.5%v/v). The lowest concentration in which inhibition occurs considered as the MIC.

GC analysis of oil sample

GC analysis of oil samples of different species of *Ocimum* was made with the help of a CE-8000 top model chromatogram using liquid nitrogen as a carrier gas. The oven temperature of the chromatogram was raised from 60° C to 220° C at the rate of 5° C/ min. The holding time of the final temperature in the oven was 10 min. The injector and detector temperature was 220° C for each. The column used for GC analysis was DB-5 MS type of capillary column of 30 mt length. The film thickness and internal diameter of the column was 25 micrometer. The concentrated essential oil samples were diluted properly upto a particular concentration using n-hexane as a solvent and one microliter of diluted sample was injected into the chromatogram for analysis (Sen &Nigam,1978). The authentic samples were also diluted similarly and the same volume was injected into the column. The peaks produced by authentic samples were compared to the peaks obtained from the test samples with respect to their retention time (RT) in order to the identification as well as quantification of the constituents present in the oil samples.

Quantitative estimation of different constituents in the essential oil

The percentage (w/w) of constituents were measured after detecting the existence of the same in the test sample by comparing the retention time of GC peaks of the test sample and that of the standard authentic ones.

Before GC analysis a stock concentration was prepared both for the authentic sample and test sample. For example, the stock concentration of authentic sample 'X' was prepared as M ppm and the concentration of test sample was prepared as N ppm. Both the stock concentration was prepared by dissolving them into HPLC, n-hexane. If the injected volume for test sample and authentic sample being 1 microliter, then the (%) of 'X' available in the test sample could be calculated by the following formula:

The concentration (%) of X in the test sample =

$$\frac{M \times A_2 \times 100}{A_1 \times N}$$

Where M = Standard stock concentration (ppm)

A_1 = Area of the standard authentic sample (obtained from chromatogram)

A_2 = Area of the test sample (obtained from chromatogram)

N = stock concentration of the test sample (ppm)

Statistical analyses

The statistical analyses of the experimental data were made with the help of SPSS software for windows.

RESULTS AND DISCUSSION

As the use of phytoextract is more advantageous than the use of synthetic compounds, their growth inhibitory action against plant pathogens is to be studied in a great detail in order to develop effective plant disease management stretegies, exploiting the available plant resources. In our present study the leaf extract of different species of *Ocimum* have been proved to be inhibitory in nature for the

Table1 : Effect of different solvent extract of leaf from different species of *Ocimum* on the growth of *Pseudomonas solanacearum*

Name of the solvent used for extraction	Name of the species					
	O.adscendans	*O.tenuiflorum*	*O.gratissimum*	*O.americanim*	*O.basilicum*	*O.kilimandscharicum*
Control	0.68± 0.01^{a}	0.58±0.01^{a}	0.73±0.005^{a}	0.64±0.01^{a}	0.66±0.02^{a}	0.71±0.03^{a}
Aqueous	0.43±0.03^{bc}	0.24±0.02	0.18±0.023	0.48±0.06	0.34±0.04	0.53±0.04^{bc}
Ethanolic	0.41±0.2^{bc}	0.23±0.003^{bcd}	0.17±0.006^{bc}	0.47±0.01^{bc}	0.33±0.08^{bc}	0.52±0.09^{bcd}
Methanolic	0.43±0.04^{bc}	0.25±0.03^{bc}	0.16±0.008^{bcd}	0.46±0.01^{bc}	0.32±0.07^{bcd}	0.52±0.04^{bcd}
Pet- ether	0.42±0.33^{bc}	0.22±0.002^{bcd}	0.17±0.007^{bc}	0.45±0.03^{bcd}	0.33±0.01^{bc}	0.54±0.03^{bc}
Acetone	0.45±0.3^{b}	0.25±0.01^{bc}	0.15±0.004^{bcd}	0.46±0.04^{bc}	0.35±0.05^{b}	0.55±0.02^{b}

**Zone of inhibhition includes diameter of disc (8mm), Values are mean ± SD, Values with different subscripts represent mean comparison by Duncan's test(at 5% level of significance).*

Table2 : Effect of different solvent extract of leaf from different species of *Ocimum* on the growth of *Alternaria brassisicola*

Name of the solvent used for extraction	Name of the species					
	O.adscendans	*O.tenuiflorum*	*O.gratissimum*	*O.americanim*	*O.basilicum*	*O. kilimandscharicum*
Control	0.942± 0.012^{a}	0.875±0.007^{a}	0.992±0.013^{a}	1.24± 0.04^{a}	0.879±0.005^{a}	0.778±0.01^{a}
Aqueous	0.842±0.003^{bc}	0.712±0.002^{bc}	0.850±0.001^{bc}	0.472± 0.01^{bc}	0.623±0.001^{bc}	0.612±0.03^{bc}
Ethanolic	0.846±0.004^{bc}	0.715±0.001^{bc}	0.842±0.012^{bc}	0.483±0.004^{b}	0.643±0.002^{bc}	0.605±0.004^{bc}
Methanolic	0.856±0.009^{bc}	0.725±0.003^{b}	0.849±0.006^{bc}	0.471±0.005^{bc}	0.639±0.004^{bc}	0.608±0.002^{bc}
Pet- ether	0.852±0.002^{bc}	0.718±0.004^{bc}	0.843±0.004^{bc}	0.481±0.001^{bc}	0.622±0.003^{bc}	0.632±0.014^{b}
Acetone	0.858±0.006^{b}	0.719±0.001^{bc}	0.852±0.005^{b}	0.474±0.007^{bc}	0.644±0.003^{b}	0.614±0.023^{bc}
Average%of inhibition	9.76	17.94	14.61	61	27	20

**Zone of inhibhition includes diameter of disc (8mm), Values are mean ± SD, Values with different subscripts represent mean comparison by Duncan's test(at 5% level of significance).*

Table 3 : Antimicrobial activity of essential oil extracted from different species of *Ocimum*

Test samples (essential oil)	Diameter of the inhibition zone for *Pseudomonas solanacearum*	Diameter of inhibition zone for *Alternaria brassisicola*
Ocimum adscendans	7.5± 0.06^{d}	5.1± 0.08^{f}
O. tenuiflorum	9.5± 0.09^{c}	8.4± 0.05^{d}
O.gratissimum	*18.7±0.13*^{a}	7.8±0.07^{e}
O.americanum	6.5 ± 0.80^{e}	*19.5±0.14*^{a}
O.basilicum	12.5± 0.11^{b}	11.6± 0.12^{b}
O.kilimandscharicum	5.4± 0.08^{f}	9.4± 0.12^{c}
Streptomycin	20± 0.12	xxxxx
Micronazole	xxxxx	22.5± 0.13

**Zone of inhibhition includes diameter of disc (8mm), Values are mean ± SD, Values with different subscripts represent mean comparison by Duncan's test(at 5% level of significance).*

growth of both wilt causing pathogenic bacteria(*Pseudomonas solanacearum*) as well as the fungal pathogen (*Alternaria brassisicola*) causing black spot disease of Crucifer. The different solvent extract used to study the growth inhibition of the pathogen shows significant difference from the control (Table 1&2). Therefore, it could be concluded that the leaf extract of different species of *Ocimum* have both antibacterial and antifungal properties. As significant difference in the growth inhibition among the different solvent extract was not recorded, it could be emphasized that some bioactive principles are common in the extract and the numerical difference among them denotes the existence of some specific constituents in the extract. The essential oil extracted from the leaf of all six species however reveals significant difference with respect to growth inhibition of the test organ-

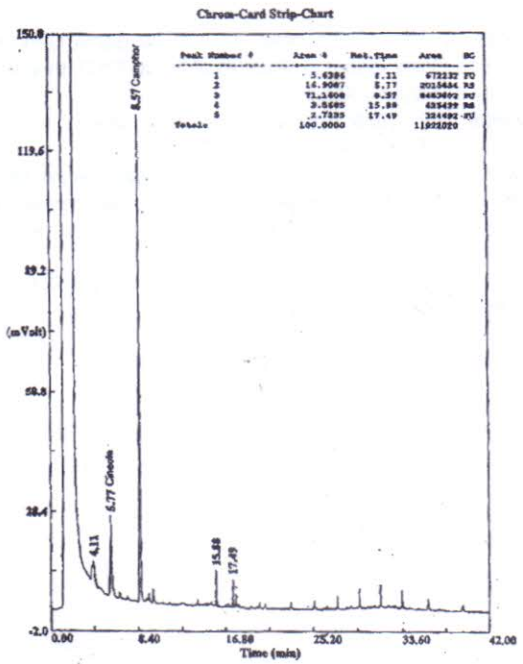

Fig. 1 : Gas Liquid Chromatogram of the essential oil sample of *Ocimum amerieamum*

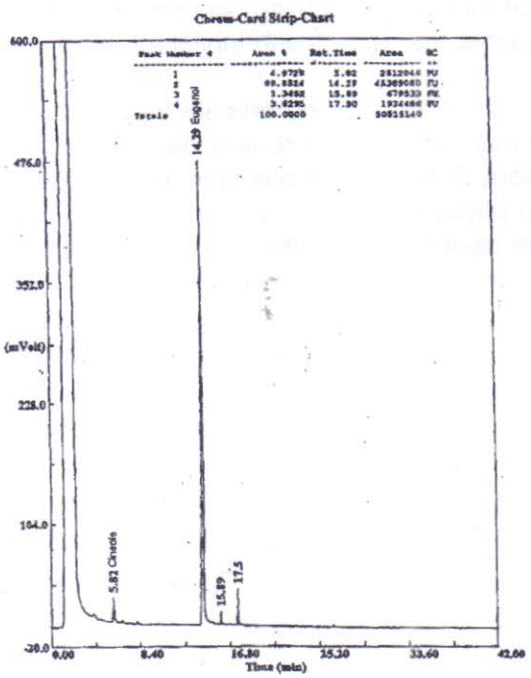

Fig. 1 : Gas Liquid Chromatogram of the essential oil sample of *Ocimum gratissmum*

ism under study. Highest radial inhibition zone formation was recorded in presence of *O. gratissimum* oil for the test organism *Pseudomonas solanacearum.* The diameter of the inhibition zone was maximum in case of *Alternaria brassisicola* due to the presence of essential oil of *Ocimum americanum* (Table 3).

The constituents of the essential oil of this two species were analysed through GLC method, which clearly reveals that the major constituent of *O. gratissimum* oil is eugenol (47.48%) whereas camphor is the major constituent of *O. americanum*(8.92%) (Fig. 1&2). In all other species the eugenol content was estimated and detected, except *O. kilimandscharicum,* where eugenol is entirely absent. Among all the species, the highest percentage of eugenol however, was recorded in the oil sample of *O. gratissimum.* The authentic sample of the major constituent when applied against the test organism, it also shows the same inhibitory effect. So, it is confirmed that the major constituent in the essential oil is growth inhibitory in nature. Thus maximum percentage of available eugenol in the essential oil may be responsible for maximum growth inhibition of *P. solanacearum.* The inhibitory effect of the essential oil of *O. kilimandscharicum* against the same test organism may be due to other different constituents in absence of eugenol. In fungal system however, such correlation was not realized. Therefore the maximum growth inhibition of *Alternaria brassisicola* in presence of essential oil from *O. americanum* may be due to camphor in addition to the low percentage of eugenol and others. The MIC of the essential oil of *O. gratissimum* against *P. solanacearum* was determined as 3% (v/v), whereas the same for the essential oil of *O. americanum* against *Alternaria brassisicola* was 4.5 %(v/v).

CONCLUSION

Thus, among all the available species of *Ocimum* growing under Indian agroclimatic condition, the essential oil of *O. gratissimum* and *O. americanum* may be considered as most potential agent to inhibit the growth of *Pseudomonas solanacearum and Alternaria brassisicola* respectively. Our study also indicates that the mode of action of the heterogeneous constituents and their availability in the essential oil of *O. gratissimum* and *O. americanum* is different for prokaryotic and eukaryotic system to exert their inhibitory effect on test organisms.

Thus there is a need of thorough phytochemical study of various species of *Ocimum* regarding its constituents that are growth inhibitory in nature.

Nevertheless, our finding reveals that apart from herbal medicine use, there is a distinct possibility to explore *Ocimum* as a potential biocontrol agent against phytopathogen. In such case dried leaves may be used to amend the soil to retard the survival of soil borne phytopathogens.

REFERENCES

Banerjee, S. 1996. Taxonomic Revision of Some Genera of the Tribe Ocimoidae (Lamiaceae) of India. Ph.D. thesis, University of Kalyani, Kalyani, Nadia.

Clevenger, J.F., 1928. Approaches for determination of volatile oil, *J. Ann. Pharm. Asso,* **17**: 346.

Daswani, L. and Bhora, A. 2002. *Science and Culture*, **68**:99-100.

Lal, R.N., Sen, T.K. and Nigam, M.C., 1978. Gas chromatography of the essential oil of *Ocimum sanctum* L. Perfume. *Kosmet.*, **59** : 230-231.

Evans, W.C., 2002. Volatile oils and resins (Chapter-23), In: Pharmacognosy, 15th edition,SAUNDERS, p.253.