PRODUCTION OF VOLATILE ANTIBIOTICS FROM AN ENDOPHYTIC FUNGUS *(PHOMOPSIS* SP.) OF *ODONTOGLOSSUM* SP. (ORCHIDACEAE) AND ITS POTENTIAL PRACTICAL APPLICATIONS

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NATIONAL FUNGAL CULTURE COLLECTION OF INDIA

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Mission

- To contribute to the knowledge of fungal biodiversity through field exploration, collection and taxonomic studies
- To provide long-term preservation of fungi as genetic resource available for research, reference and exploitation
- To provide services to researcher, industry and society related to fungal biology
- To disseminate the knowledge through training programmes, organising conferences, publications in monographs, technical bulletin & scientific journals

ENDOPHYTES-SOME FACTS

- **Endophytes represent an enormous diversity and their specialized habituations make them a stimulating field of study.**

- **Other than mere diversity research attention is now paid to the bioactive metabolites of endophyte.**

- **The fact that endophytes can make some phytochemicals originally characteristic of the host has changed the scope of endophytic biology and the importance of the output of endophytes.**

- **Different classes of natural products have been discovered from endophytes. (antibacterial, antifungal, antitumor, antiviral & industrial enzymes etc.; even unusual molecules that act as antidiabetic, immunomodulatory, herbicidal, and plant growth promoting/plant protective agents).**

- **Recent report of production of volatile organic compounds (VOCs) with the various use in agriculture by endophyte has open a new path and proved that endophytes produce an abundant and reliable source of novel natural products and are still poorly understood.**

METHODS

- **Isolation of endophytes [selective]**
- **Biological screening [plate assay]**
- **Qualitative analyses of VOCs [GC/MS]**
- ***Bioactivity of artificial mixture of VOCs***
- **Quantitative analysis of VOCS [PTR-MS]**
- **Characterization & identification[Polyphasic]**
- **Conservation & application**

- **More than 100 isolates of fungi and actinomycetes isolated into pure cultures from leaf and stem tissues of 12 plants collected from different locations of cloudy forests of Ecuador.**

- **Based on *in vitro* cultural and morphological criteria isolates were categorized in to 26 different strains of fungi and 4 strains of actinomycetes.**

- **In screening plates containing water agar medium (WA), 5 colonies appeared growing from sterilized leaf tissues of *Odontoglossum* sp. (Orchidaceae), 4-days after incubation.**

- **These colonies were found identical in culture morphology, growth pattern and producing an interesting aeromatic smell were assigned number 'EC-4'and selected for the bioactivity tests.**

Odontoglossum from Ecuador

some endophytes

some endophytes

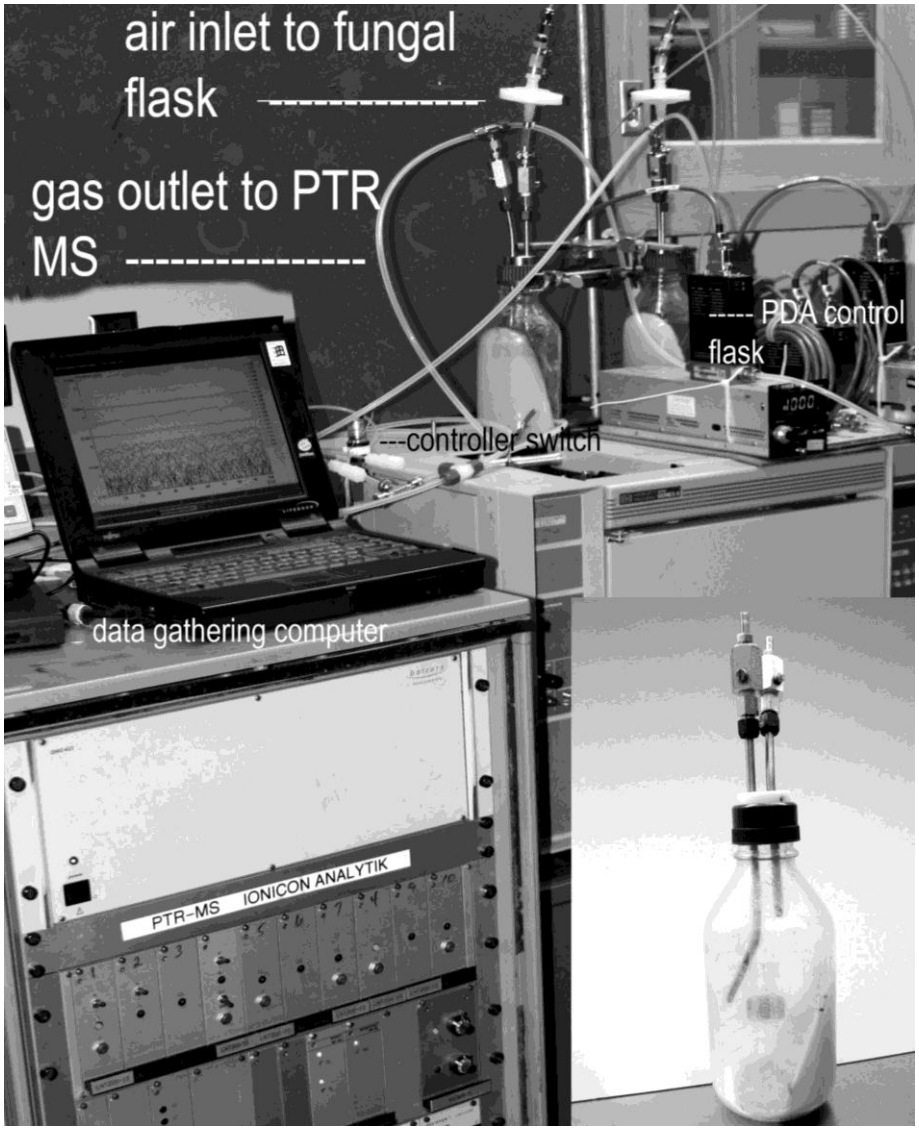
Test organism	Inhibition (%) after 144 h exposure*	IC_{50} of artificial atmosphere after 48 h (µl ml^{-1})	Concentration in µl/ml of air space required to produce 50% reduction
Aspergillus fumigatus	57.00 ± 0.5773	20.00 ± 1.15	0.400
Botrytis cinerea	37.83 ± 0.5773	11.50 ± 0.11	0.230
Ceratocystis ulmi	11.13 ± 1.5275	13.80 ± 0.00	0.276
Cercospora beticola	19.59±0.5773	10.40 ± 0.00	0.208
Colletotrichum lagenarium	No inhibition	14.40 ± 0.00	0.288
Fusarium solani	43.22 ± 0.0000	22.60 ± 1.61	0.452
Geotrichum candidum	45.36 ± 0.5773	25.65 ± 0.99	0.513
Phytophthora palmivora	5.66 ± 0.5773	11.76 ± 1.77	0.235
Pythium ultimum	59.11 ±0.957	08.00 ± 0.80	0.160
Pytophthora cinnamomi	42.04 ±0.5773	12.80 ± 0.34	0.256
Rhizoctonia solani	53.00 ±1.000	20.72 ± 0.95	0.414
Sclerotinia sclerotiorum	70.78 ±1.1547	14.49 ± 0.35	0.289
Trichoderma viride	No inhibition	14.50 ± 0.69	0.290
Verticillium dahliae	19.42 ± 0.000	14.40 ± 0.00	0.288

Effect of 7-day old culture of *Phomopsis* sp. (EC-4) VOCs on selected fungal pathogens. Inhibition values calculated as a %age of growth inhibition relative to a control test organism. Concentration of artificial mixture of VOCs in µl/ml of air space required to produce 50% reduction were calculated. Tests were conducted in triplicate and results varied as indicated by standard deviations.

RT (min:s)	Total area (%)	Possible compounds	Stds*	MW	Quality
2.52	1.11	2-Propanone		58	64
6.39	1.50	Benzene, methyl		92	91
7.10	1.21	**Unknown****		132	
7.28	0.90	**Unknown****		164	
7.53	1.21	**Unknown****		194	
7.84	2.80	1-Propanol, 2-methyl-	*	74	91
8.50	4.72	Benzene, ethyl-		106	95
10.65	5.22	Sabinene	*	136	91
10.88	105.17	1-Butanol, 3-methyl	*	88	83
20.58	1.39	**Unknown****		162	
22.36	2.24	(+ -) – gymnomitrene		204	64
23.35	7.68	(-) -.beta.-Acoradiene		204	64
24.84	4.46	**Unknown****		133	
28.34	8.76	Benzeneethanol	*	122	91
41.61	1.11	**Unknown****		294	59

*** Retention time and MS spectrum closely matched or were identical to an standard compound. Those compounds without a designated foot note have a mass spectrum that most closely matched the appropriate compound in the NIST database.** Unknown compounds represent those with a quality value less than 50.**

Selected Key compounds from *Phomopsis* (EC-4)



PROTON TRANSFER-MASS SPECTROMETER (PTR-MS)

PTR-MS instrument ionizes organic molecules in the gas phase through reaction with H_3O^+, forming mostly protonated molecules (MH^+)

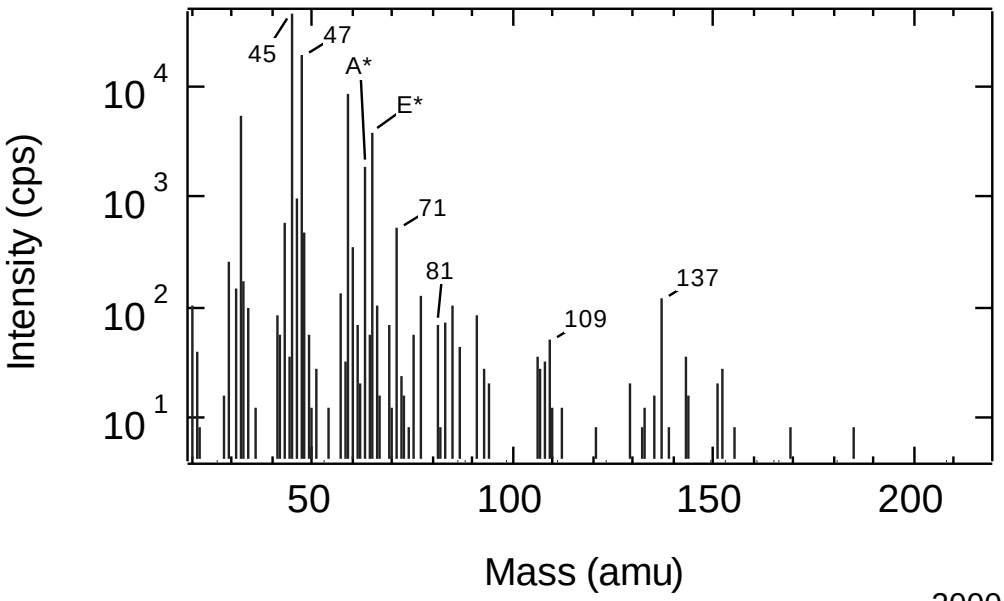
which can be detected by a standard quadrupole mass spectrometer.

This process can be run on real air samples with or without dilution, since the primary constituents of air (nitrogen, oxygen, argon and carbon dioxide) have a proton affinity less than water and thus are not ionized.

Most organic molecules (excepting alkanes) have a proton affinity greater than water and are therefore ionized and detected.

Enormous advantage of PTR-MS is that it can be run in real time and continuously produce data on the concentrations of specific ions of interest.

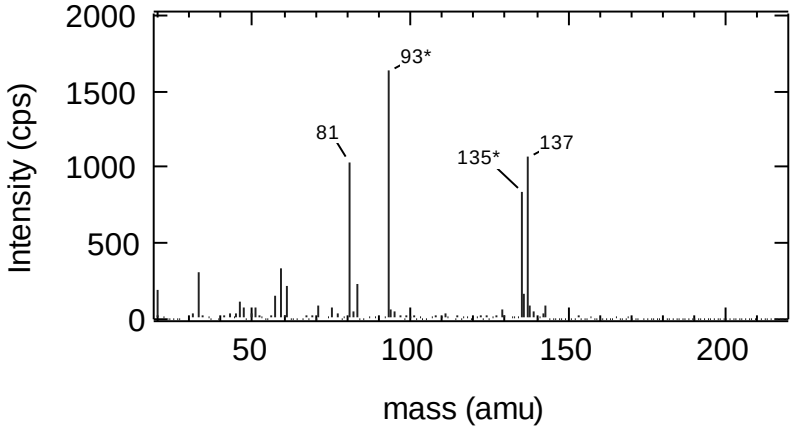
***Lindinger W, Hansel A, Jordan A (1998) On-line monitoring of volatile organic compounds at pptv levels by means of proton-transfer-reaction mass spectrometry (PTR-MS): medical applications, food control and environmental research. Int J Mass Spectrometry Ion Process 173:191–241**



-Sabinene - m/z 81 & m/z 137
3-methylbutanol - m/z 71

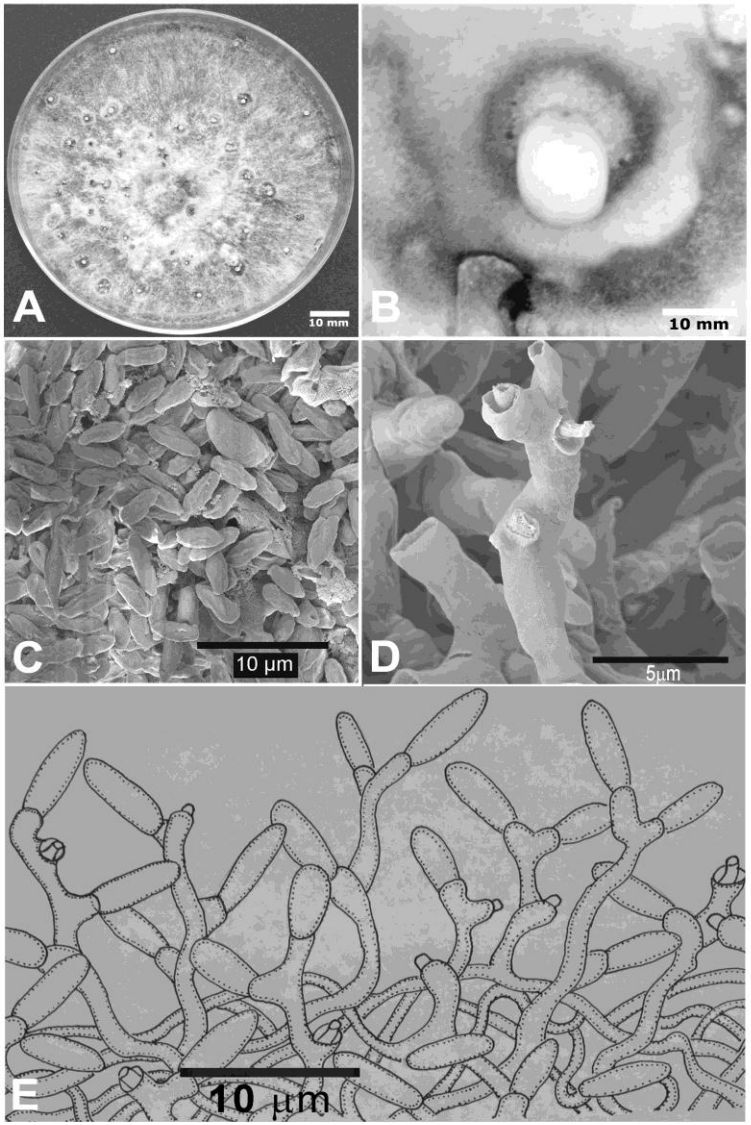
-The total volatile production measured was 18.4 ppm

1



2

1. A PTR MASS SPECTRUM OF A 7 DAY OLD CULTURE OF. *PHOMOPSIS* SP. (EC-4).
2. A PTR MASS SPECTRUM OF A STANDARD SABINENE OBTAINED FROM SIGMA-ALDRICH.



A-E: ***Phomopsis*** **sp. (EC-4)**

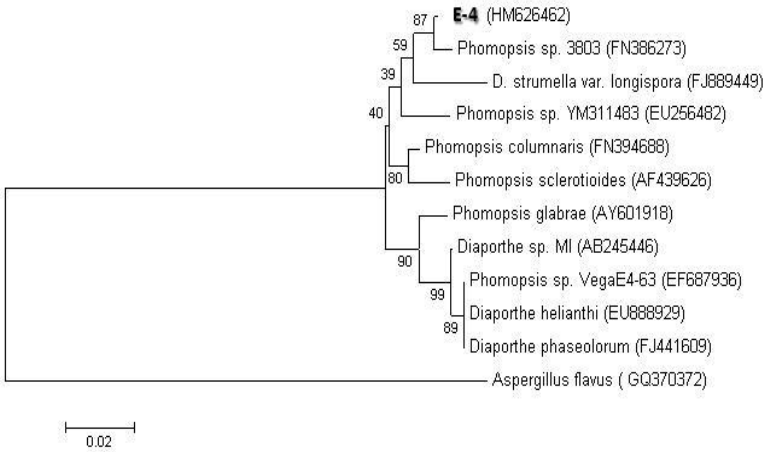
A. Colony with fruiting on PDA

B. Oozing of slimy mass of conidia from a conidioma developed on PDA

C. Scanning electron micrographs of alpha conidia

D. Scanning electron micrographs of conidiophores with condiogenous loci (scars)

E. Line drawing showing conidia attached from conidiophores arising out from fragile vegetative mycelia growing *in vitro*



Neighbour joining tree based on ITS1-5.8S-ITS2 sequences showing the relationships among species of *Phomopsis* and *Diaporthe*.

Acc.No. MSU (EC-4) 2377

HM626462-http://www.ncbi.nlm.nih.gov

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ACHIEVEMENTS

- **Isolation and identification of an unusual species of *Phomopsis* (EC-4), produces a distinct set of volatile compounds**
- **VOCs showed antifungal activity against wide range of plant pathogens**
- **Artificial mixture of volatiles mimicked the antibiotic effects of *Phomopsis* sp. (EC-4)**
- **Tests on 2-34 µl of the artificial gas mixture per 50 ml of air space in PDA plate revealed IC_{50} value between 8–25.65 µl/mL**
- **Olfactory tests revealed media enriched with starch, glucose, and cellobiose facilitates higher concentrations of volatile compounds**
- **PTR-MS revealed concentration of total VOCs emissions as 18.4 ppm.**
- **This is the first report of fungal sabinene**
- **Volatiles produced by known *Muscodor albus* did not affect the *Phomopsis* sp.**

OUTLOOK & GOALS

- Selection of hosts from unusual habitats or niches
- Selective Isolation of endophytes from selected hosts
- Selection of bioactive endophytes
- Screening of endophyte producing VOCs
- Selection of VOCs as mycofumigant
- Screening of endophytes for biofuel production
- VOCs as alternative of agrochemical to be phased out
- Use of advance tools for future research on endophytes

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