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USE OF FT-IR SPECTROSCOPY IN TAXONOMIC RELATIONSHIPS OF CERTAIN MUCORACEOUS FUNGI: A PRELIMINARY STUDY

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Abstract

Fungal classifications based on morphological and cultural characteristics are accepted as standard method in fungal taxonomy. Fungal classifications upto last two decades or so were solely based on morphological characteristics, but with the continuous advancement of molecular techniques, taxonomists shifted their approach towards molecular analysis of taxa to classify. However, these advance and sophisticated techniques have their own limitations. Therefore, a combined polyphasic approach combining molecular taxonomy with biochemical profiling is preffered. In the present study we tried to use FT-IR spectroscopy as a tool to establish the relationship between different isolated mucoraceous species and to distinguish between different strains, within species. The fuctional groups present on the fungal biomass, as observed by FT-IR spectroscopy, were used to assess the similarity coefficient between fungal taxa studied.

***Keywords*:** Mucoraceous fungi, FT-IR spectroscopy and similarity coefficient.

Introduction

Zygomycota is one of the four phyla of Fungi recognized in the Dictionary of the Fungi (9th Edition: Kirk *et al.*, 2001). Fungi under this group are commonly found in soil, dung, organic debris, air as saprophytes and few as biotrophs on plants and animals. With the ongoing use of molecular information in fungal systematics, the classification of fungi is in a 'flux' and prone to drastic revisions. Hibbett *et al*. (2007) based on the molecular phylogeny disintegrated zygomycota and replaced it by four subphyla Entomophthoromycotina, Kickxellomycotina, Mucoromycotina and Zoopagomycotina. The subphylum Mucoromycotina (Benny *et al.,* 2016) includes two orders Endogonales and Mucorales. Over a period of time a lot of merger, rearrangement, derecognition of many mucoralean species and erection of new taxa has taken place (Upadhyay and Charaya, 2009).

Classifications based on morphological characteristics are historically accepted as standard method in fungal taxonomy. However, sometimes morphological characteristics do not reflect phylogenetic relationships. Therefore, taxonomical study, which relies on molecular information is preferred. Schoch *et al*. (2012) proposed use of nuclear ribosomal internal transcribed spacer region (ITS) as a universal DNA barcodes to fungal identification. However most unidentifiable ITS sequences on Gene Bank belong to kingdom Fungi, making molecular approach quite limited (O'Brien *et al.,* 2005). Moreover, these molecular techniques are quite complicated, often requiring sophisticated highly expensive equipment and chemicals. Therefore, a combined polyphasic approach combining molecular taxonomy with biochemical profiling is advantageous (Frisvad *et al*., 2008; Stadler *et al*., 2010).

Fourier transform-infrared (FT-IR) spectroscopy is a biophysical method that has came out as a routine analysis for rapid characterization and identification of microorganisms (Shapaval *et al*., 2013, 2014). FT-IR has been used successfully for the identification of filamentous fungi and yeasts− their genera and species with a high degree of confidence (Kummerle *et al*., 1998; Nauman *et al*., 2005; Fischer *et al*., 2006). It has been successfully used for differentiating strains of *Aspergillus* and *Penicillium* (Fischer *et al*., 2006), strains of *Candida* (Sandt *et al*., 2003; Essendoubi *et al*., 2005). Remarkable variations do exist amongst the fungi with respect to their cell wall composition and organization that has turned out to be of great taxonomic value (Bartnicki Garcia, 1968; Ruiz-Herrera, 1977). To study these variations among chemical functional groups in the cells, vibrational spectroscopy has emerged as a promising tool for obtaining a clearer picture of these variations. In the present study the FT-IR spectra of different isolated mucoraceous strains were studied to distinguish between different strains and to get a better insight into their taxonomic relationship.

Materials and Methods

Collection of soil samples

Samples were collected from soils, air and other resources including rotting fruits covering six districts of western U.P., namely Muzaffarnagar, Meerut, Baghpat, Shamli, Hapur and Ghaziabad. A total of thirty soil samples, five soil samples

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from each of the six districts, were taken from apparently non-polluted agricultural lands. The surface layer of the soil was removed with the help of a trowel to remove extraneous litter/organic matter. Soil samples were then collected aseptically and brought to the laboratory for the isolation and further experiments. To sample air mycobiota, five Petri plates (each containing 20 ml cooled and sterilized PDA medium with 0.03 g of Rose Bengal) and five Petri plates (each containing 20 ml cooled and sterilized Czapek's Dox Agar medium) were exposed to air for five min. at different locations in Meerut. Several rotting fruits (apple, banana, brinjal, luffa, tomato) were also collected from fruit and vegetable markets of Meerut. These samples were brought to the laboratory for further experiments.

Isolation of fungi from soils

All the five soil samples collected from the each district were mixed thoroughly to obtain one composite sample, thus obtaining six composite soil samples. Culturing was carried out on Potato Dextrose Agar medium (Thom and Raper, 1949) using serial dilution method. The Petri dishes (in triplicate) containing medium (20 ml of cooled and sterilized PDA with 0.03 g of Rose Bengal) and the inocula (one ml aliquot) were incubated at 25±1°C for 3-8 days.

Isolation from air samples

Ten air samples taken from random locations in Meerut city, including (i) five air-exposed Petri plates, containing 20 ml cooled and sterilized PDA medium and (ii) five air exposed Petri plates, containing 20 ml cooled and sterilized Czapek's Dox Agar medium, were incubated at 25±2°C for 3-8 days. All experiments were run in triplicate.

Isolation from rotting fruits

Different spoiled fruits collected, were incubated for isolation of the associated mycobiota using moist chamber technique. The sample of fruits were placed on a layer of moist filter paper and covered with flask in order to prepare a moist chamber. The fruits were then allowed to rest at 25±2°C for 3-8 days. The fungal growth were transferred to Petri plates, containing 20 ml of cooled and sterilized PDA medium as well as Petri plates containing 20 ml of cooled and sterilized Czapek's Dox Agar medium. All Petri plates were incubated at 25±2°C for 3-8 days.

Records of fungi isolated

A complete record of fungal species and their numbers in each case was maintained. The identification of the fungal species was done on the basis of their morphology and cultural characteristics following Gilman (1957), Tandon (1968), Ellis (1971, 1976), Subramanian (1971), Hesseltine and Ellis (1973), Domsch *et al.* (1980) and Nagamani *et al*. (2006). The total number of colonies and the number of colonies of individual fungal species growing were recorded.

Preparation of fungal biomass

Biomass of selected mucoraceous strains was prepared for FT-IR analysis. Fungal biomass was prepared using MGYP broth. After steam sterilization at 15 lb pressure and 121^{0}C temperature for 15 minutes, 5 flasks containing MGYP broth were inoculated with the given strain from the prepared plates. The flasks were then incubated at 27±1°C on the shaker for one week. The fungal biomass was then harvested by filtration, washed with generous amounts of distilled water, resuspended in water, washed and filtered again. The wet biomass thus obtained was dried in an electric oven at 70°C for 2 days to get dry biomass.

FT-IR analysis of fungal biomass

FT-IR analysis of dry powdered biomass of selected fungal species was conducted to assess the chemical changes in biomass to assess the relationship, if any, between the functional groups on the biomass. Two mg of fungal biomass powder were mixed with 98 mg of dry powdered potassium bromide (KBr) and finally grounded. The material was used for preparing pellets by applying pressure of 10,000-15,000 psi. IR spectra of these were recorded on IR-affinity-1, Shimadzu model FT-IR spectrophotometer at high resolution (≤ 0.001 cm-1). The degree of similarity between isolated mucoraceous strains was deciphered by calculating similarity coefficient (based on absence or presence of functional groups), on the basis of which dendrograms were constructed.

Result and Discussion

Mycobiota isolated from soils and other substrates

A total of 29 fungal species were isolated from (i) soils collected from different districts of Western U.P., (ii) samples of fruits collected from fruit and vegetable markets at Meerut and (iii) cow dung; as also from air samples. Out of these, eleven taxa belonged to Mucorales, represented by four genera and nine species. The isolated mucoraceous fungi includes- (i) *Cunninghamella elegans* Lendner; (ii) *Mucor hiemalis* Wehmer; (iii) *Mucor mucedo* Fresenius; (iv) *Mucor plumbeus* Bonorden; (v) *Mucor racemosus* Fresenius; (vi) *Pilobolus crystallinus* (F.H. Wiggers) Tode; (vii) *Rhizopus arrhizus* Fischer strain 1; (viii) *Rhizopus arrhizus* Fischer strain 2; (ix) *Rhizopus microsporus* Tieghem (x) *Rhizopus stolonifer* Ehrenberg strain 1 and (xi) *Rhizopus stolonifer* Ehrenberg strain 2.

Two species of mucoraceous fungi *i.e. Mucor hiemalis* and *Rhizopus arrhizus* were obtained from air samples. Four species of mucoraceous fungi were isolated from other sources *i.e.* apple (*R. arrhizus*), banana (*R. arrhizus* and *R. stolonifer*), brinjal (*R. arrhizus*), luffa (*R. stolonifer*), tomato (*M. hiemalis* and *Rhizopus arrhizus*) and from dung (*Pilobolus crystallinus*).

FT-IR spectra of mucoraceous fungi isolated

FT-IR spectroscopy of the 10 strains of mucoraceous fungi

isolated (excluding *Pilobolus crystallinus*) was conducted. The objective was to work out the relationship between isolated fungal taxa and to evaluate the possibilities of making functional groups, the basis of establishing relationship between the strains as compared to other criteria adopted by different workers. The spectra are presented FT-1 to FT-10, while table 1 represents the consolidated list of different functional groups represented by peaks in FT-IR spectra of selected fungal biomass. On the basis of the number and types of functional groups, S_m coefficients were calculated. The value of S_m coefficient were found to be-

a) between different strains of *Mucor*-

Mucor mucedo X *Mucor plumbeus*: 0.743

Mucor mucedo X *Mucor racemosus*: 0.724

Mucor plumbeus X *Mucor racemosus*: 0.617

Mucor plumbeus X *Mucor hiemalis*: 0.783

Mucor raceosus X *Mucor hiemalis*: 0.435

b) between different strains of *Rhizopus*-

Rhizopus arrhizus strain 1 X *Rhizopus microsporus*: 0.741

Rhizopus arrhizus strain 1 X *Rhizopus arrhizus* strain 2: 0.769

Rhizopus arrhizus strain 1 X *Rhizopus stolonifer* strain 1: 0.805

Rhizopus arrhizus strain 1 X *Rhizopus stolonifer* strain 2: 0.654

Rhizopus microsporus X *Rhizopus arrhizus* strain 2: 0.781

Rhizopus microsporus X *Rhizopus stolonifer* strain 1: 0.833

Rhizopus microsporus X *Rhizopus stolonifer* strain 2: 0.851

Rhizopus arrhizus strain 2 X *Rhizopus stolonifer* strain 2: 0.696

Rhizopus arrhizus strain 2 X *Rhizopus stolonifer* strain 1: 0.764

Rhizopus stolonifer strain 1 X *Rhizopus stolonifer* strain 2: 0.650

c) between the genera *Mucor*, *Rhizopus* and *Cunninghamella*-

Mucor X *Rhizopus*: 0.8125

Mucor X *Cunninghamella*: 0.622

Rhizopus X *Cunninghamella*: 0.761

Phylogenetic relationships between isolated taxa

On the basis of above S_m coefficient the dendrograms between different species of *Mucor*, different species of *Rhizopus* and between the genera *Mucor*, *Rhizopus* and *Cunninghamella* were constructed (Fig. 1). From the dendrograms, it is clear that the genus *Mucor* and *Rhizopus* are more closely related than to *Cunninghamella* which forms a separate clade.

Voigt and Kirk (2014) have worked out the family structure of Mucorales, based on phylogenetic analysis of aligned nucleotide sequence encoding 15 S and 28 S subunit ribosomal RNA and exonic regions of actin and translational elongation factor 1α. They also found Mucoraceae and Rhizopodaceae to be closer than Cunninghamellaceae on a branch separate from Mucorineae which bears Rhizopodaceae and Mucoraceae. Voigt and Wostemeyer (2001) determined sequence of nuclear encoded gene actin (act) and translation elongation factor EF-1α and classified Mucorales on the basis of these features. They found *Mucor hiemalis* to be quite close to *M. mucedo* and quite distant from *M. racemosus*. In the present study also (Fig. 1) *M. hiemalis* and *M. mucedo* are closer to each other, as compared to *M. racemosus*. Abe *et al.* (2010) constructed phylogentic tree of *Rhizopus* on the basis of EF-1α where *R. microsporus* is closer to *R. stolonifer* than *R. oryzae* (*R. arrhizus* strain 2). Earlier Voigt and Wostemeyer (2001) found *R. microsporus* closer to *R. oryzae* than to *R. stolonifer*. The results of the present study reveal that *R. microsporus* and both the strains of *R. stolonifer* are closer than to other two other strains of *R. arrhizus*, thus supporting the tree constructed by Abe *et al.* (2010).

It is clear from the above discussion that FT-IR spectroscopy of fungal biomass can definitely help in resolving phylogenetic relationships between different taxa. No study on phylogenetic relationships between taxa on the basis of FT-IR spectroscopy was available for comparison. However, more studies with more taxa need to be conducted before establishing FT-IR spectroscopy as a taxonomic tool.

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Table 1: Functional groups and number of peaks of biomass of different selected fungal taxa

S. No.	Functional Group	Fungal Taxa									
		Mucor mucedo	*Mucor plumbeus*	*Mucor racemosus*	*Mucor hiemalis*	*Rhizopus arrhizus* 1	*Rhizopus microsporus*	*Rhizopus arrhizus* 2	*Rhizopus stolonifer* 1	*Rhizopus stolonifer* 2	*C. elegans*
1.	Alcohol	-	-	-	1	-	-	-	-	-	-
2.	Alkyne	-	1	-	-	1	-	1	-	-	-
3.	Amide	2	1	-	1	1	-	-	1	-	-
4.	Amine	2	1	-	1	1	-	-	1	-	-
5.	Aromatic C-H	2	3	-	4	1	1	2	1	-	2
6.	Aromatic rings	2	1	2	1	2	1	2	2	1	2
7.	C=S	4	1	2	1	2	1	4	2	1	3
8.	Carboxylate salt	1	-	-	1	-	-	-	-	-	-
9.	C-C aliphatic chain	2	2	3	-	5	2	5	2	1	4
10.	C-CH_3	3	3	1	3	3	2	3	2	1	3
11.	C-Cl	1	-	-	-	-	-	1	-	-	1
12.	C-F	1	-	-	-	-	-	1	-	-	1
13.	CH_2	1	1	1	2	1	1	2	1	1	1
14.	=CH_2	-	1	-	2	1	-	-	-	-	-
15.	C-O-C	-	-	-	2	2	-	-	-	-	-
16.	Diazonium salt	1	-	-	1	-	-	-	-	-	-
17.	Isothiocynate	-	-	-	-	1	1	1	-	-	-
18.	Nitro	1	-	-	-	-	-	-	-	-	-
19.	O-CH_3	1	-	-	-	-	-	1	-	-	-
20.	OH	5	4	-	4	2	1	1	2	1	2
21.	P-H	2	1	1	2	-	-	-	-	-	-
22.	Phenol	1	1	-	1	1	-	-	-	-	-
23.	Si-O-C	3	1	2	1	2	1	3	2	2	2
24.	Si-O-Si	2	1	2	1	2	1	2	2	2	2
25.	Sulfonamide	2	1	1	1	1	1	2	1	-	1
26.	Sulfone	2	1	1	1	1	1	2	1	1	1
27.	Sulfonic Acid	2	-	1	-	1	-	3	1	1	2
28.	Thiocynate	-	-	-	-	1	1	1	-	-	-

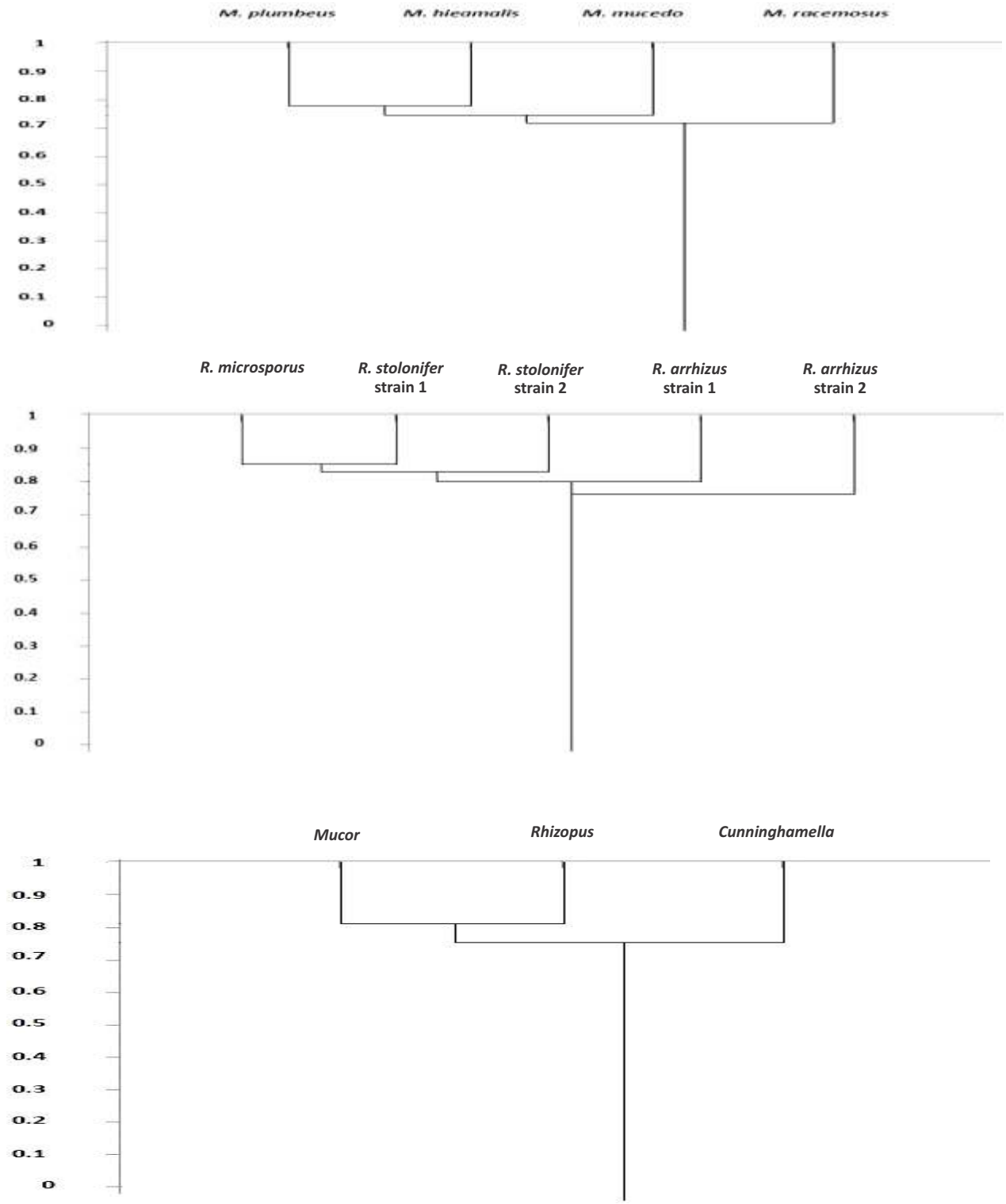

Fig 1: Dendrograms showing relationships between different taxa of Mucorales on the basis of FT-IR spectroscopy