Polyphasic taxonomy for species delineation of genus *Penicillium*

जीनस पेनिसिलियम के प्रजाति निरूपण हेतु बहु अवस्थावर्गीकरण विज्ञान

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Polyphasic taxonomy for species delineation of genus *Penicillium*

A Thesis

By

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CERTIFICATE

This is to certify that the thesis entitled "**Polyphasic taxonomy for species delineation of genus *Penecillium***" submitted to the Faculty of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfillment of the requirements for the award of the degree of **Master of science in Plant Pathology** is a record of bona fide research work carried out by **Mr. Rubin Debbarma, Roll No. 20587** under my guidance and supervision and that no part of this thesis has been submitted for any other degree or diploma. I further certify that any help or information received during the work on this thesis has been duly acknowledged.

Place: New Delhi

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LIST OF ABBREVIATIONS

ºC	Degree Centigrade
kg	Kilogram
L	Liter
min	Minutes
h	Hour
g	Gram
ml	Milliliter
cm	Centimeter
mm	Millimeter
µg	Microgram
µl	Microliter
ng	Nanogram
Pmol	Picomol
mM	Millimolar
M	Molar
Conc.	Concentration
ITCC	Indian Type Culture Collection
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
MEA	Malt Extract Agar
OMA	Oat Meal Agar
CYA	Czapek Yeast Autolysate Agar
rpm	Revolution per minute
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleotide 5' triphosphate
PCR	Polymerase Chain Reaction
Et Br	Ethidium bromide
CTAB	Cetyl Trimethyl Ammonium Bromide
bp	Base pair
β-tubulin	Beta-tubulin
MP	Maximum Parsimony
BLAST	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information
PCI	Probability of Correct Identification
SM	Secondary metabolites
TLC	Thin Layer Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
HPLC	High Performance Liquid Chromatography
RT	Retention Time

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Penicillium is one of cosmopolitan fungi present in the different habitats such as soil, plants, air, covered environments and several food items. Due to its worldwide distribution, it impacts economically on human life. Several species of the genus *Penicillium viz., Penicillium camemberti* and *P. roqueforti* play a central role in cheese industries. *P. nalgiovense* is being used for taste enhancement of sausages and also check colonization by other fungi and bacteria (Thom, 1906, Nelson, 1970, Karahadian *et al*., 1985, Giraud *et al*., 2010). In addition, *Penicillium* species produce of a number of macromolecules (gluconic, citric and tartaric acids) and several enzymes (pectinases, lipase, amylases, cellulases, and proteases). Some *Penicillium* species are high impact in bioremediation due to decomposition of xenobiotic compounds. Many species of *Penicillium* are source of major antibiotics especially Penicillin is produced by *P. chrysogenum* which inhibits the growth of Gram-positive bacteria.

Penicillium species are also known to produce mycotoxins which are highly toxic. Their ability to grow on seeds and other preserved foods shows their tendency to survive in low humidity and when the seeds are sufficiently moist, cause devastating rots in food crops as pre- and postharvest pathogens (Frisvad and Samson, 2004, Pitt and Hocking, 2009, Samson *et al.*, 2010).

The genus *Penicillium* is an ascomycetous fungus belongs to the family *Trichomaceae* which currently contains 354 accepted species. The name of *Penicillium* is derived from its reproductive structure called penicillus, which means “little brush” (Link, 1809). The shape of the Penicillus may be monoverticillate, biverticillate, terverticillate or quarterverticillate. It contains fertile hyphae, stipes, rami, ramuli, metulae, phialides and conidia. The shape of phialides are ampulliform (flask shaped) and acerose (cylindrical) with long and short collula. The Conidia of *Penicillium* are sometime ornamented, pigmented green or blue and variable in shape and size. Colony characters and radial growth on specific media are important charcters for species identification. Czapek Yeast Autolysate agar (CYA) and Malt Extract agar (MEA) are recommended as standard media for the good growth of *Penicillium* and for morphological characterization.

Species concept describes not according to similarity of appearance of a species but their interbreed members of populations in nature. In *Penicillium*, species concept was used by Thom (1954) by standardizing different morphological characters in systemic manner to delineate species. It was further emphasized by Pitt (1979), Samson and Pitt (1985), Okuda (1994) and Okuda *et al.,* (2000).

The genus *Penicillium* has four subgenera namely *Penicillium, Biverticilllium, Aspergilloides* and *Furcatum.* Due to large number of species, the genus *Penicillium* is further divided into different subgenus and subsequently sections and series. Currently the genus contains five sections and seventeen series (Frisvad and Samson, 2004). Even though the *Penicillium* species are very common and the taxonomic structure of the genus is well defined, species identification is still problematic due to overlapping macro- and micro-morphological characters. The closely related species such as *P. chrysogenum* and *P. flavigenum* is difficult to identify based morphological characters alone. Thus it is essential to find an alternative method for accurate identification of the species of this genus.

Therefore the combined approach was suggested using a combination of morphological and molecular data. However, single character may not be good to discribe a new species, but applying the mant parameters in combination characters will allow describing new species. Phylogenetic analyses of single gene sequences have been used in the molecular studies of several species of *Penicillium* using ribosomal genes and protein coding genes. The ITS-rDNA region is a universally accepted barcode but has some limitations due to less intraspecific divergence in genus *Penicillium* and some other ascomyceteous fungi. Therefore, β-tubulin sequences are being used as good marker for better species-specific resolution in *Penicillium* spp. These studies have determined some species delineation, but have not provided a holistic resolution at genus level.

Taxonomy of genus *Penicillium* has been recently revised on the basis of various recent criteria i.e., the production of specific secondary metabolite. Secondary metabolite is low-molecular weight molecules but chemically diverse produced by biosynthetic pathways. Some secondary metabolites are mycotoxins like patulin which are destructive, but others are important for human health *viz.*, penicillin and griseofulvin. It is also reported that one species (*P. griseofulvum*) may produce harmful (patulin) and beneficial secondary metabolites (griseofulvin).

Therefore, study on polyphasic approach using morphology, molecular and secondary metabolite production data has been carried out to establish the species charcters of genus *Penicillium* with a title "**Polyphasic taxonomy for species delineation of genus *Penicillium***" and the following objectives:

1. Morphological and molecular characterization of *Penicillium* species.
2. Chemo-profiling of *Penicillium* species and their taxonomical evaluation using morphological, molecular and secondary metabolic data.

The genus name *Penicillium* is derived from its reproductive structure called penicillus, which means small brush (Link, 1809). Its main function in nature is the remediation of organic wastes, whereas some species also cause a range of pre- and postharvest rots in agriculturally important crops (Samson *et al.,* 2010), *Penicillium* species are also known to produce adverse range of mycotoxins (Frisvad *et al.*, 2004). Some species have positive impact with the food industry for the production of cheeses (Camembert or Roquefort) and fermented sausages (Giraud *et al.*, 2010, Lopez- Díaz *et al*., 2001, Ludemann *et al.,* 2010). Their degradative ability has resulted in species being screened for the production of novel enzymes (Raper and Thom, 1949, Li *et al.*, 2007, Adsul *et al*, 2007, Terrasan *et al.*, 2010). Its biggest impact and claim to fame is the production of penicillin, which revolutionized medical approaches for treating bacterial diseases (Fleming, 1929, Chain *et al.,* 1940, Abraham *et al.,* 1941, Thom, 1945). Many other extrolites have since been discovered that are used for a wide range of applications (Frisvad *et al.,* 2004). Pitt (1979) considered it axiomatic that *Penicillium* or one of its products has affected every modern human.

2.1 Ecology and distribution of *Penicillium*

All species of *Penicillium* are capable to grow at 25^{o}C but some may also grow at 5ºC and some are growing well at 15ºC. Most of the species in *Penicillium* are associated with pre and post-harvest diseases of agriculturally important crops (Thom, 1930, Raper and Thom, 1949, Pitt, 1979). All Penicillia can be grown on artificatial media in laboratory, but they may grow well on autoclaved cereal based laboratory media and do not require special media (Pitt, 1979).

2.2 Nomenclature of genus *Penicillium*

It is now more than 200 years since Link (1809) introduced the generic name *Penicillium* and described the three species *P. candidum, P. glaucum* and the generic type *P. expansum.* Since then, more than 1000 names were introduced in the genus. Many of these names are not recognizable today because descriptions were incomplete by modern criteria. Some names were published invalidly, or are now considered synonyms of other species. Thom (1930) revised all species described

until 1930 and accepted 300 species. In later studies, Raper and Thom (1949) accepted 137 species, Pitt (1979) accepted 150 species, and Ramírez (1982) accepted 252 species (numbers include species described in *Eupenicillium*). At that time, a morphological species concept was used for *Penicillium* classification and identification. Using DNA sequencing was started during 1990's. So DNA sequencing created the threat for old names previously considered for uncertain application, because their ex-type cultures were no longer morphologically representative and replaced by new names. The List of "Names in Current Use" (NCU) for the family *Trichocomaceae* (Pitt and Samson, 1993) accepted 223 species by deleting not published names. This list was updated by Pitt *et al.* (2000) who accepted 225 species. Species names not accepted on these lists were not to be disregarded permanently, as stated by *Pitt et al.*, (2000), because they were not formally rejected under the nomenclatural code and could still be reintroduced in a revised taxonomy. In fact, this became common practice as many old species were shown to be distinct and were reintroduced (Peterson *et al.*, 2005, Serra *et al.*, 2008, Houbraken *et al*., 2011a,b, Houbraken *et al*., 2012a, Visagie *et al.*, 2013).

The abandonment of article 59 in the new International Code of Nomenclature for algae, fungi and plants (ICN) (McNeill *et al*., 2012) resulted in single name nomenclature for fungi. In anticipation of this change, Houbraken and Samson (2011) redefined the genera in the family *Trichocomaceae* based on a four gene phylogeny. They segregated the *Trichocomaceae* into three families, namely the *Aspergillaceae* (*Aspergillus, Hamigera, Leiothecium, Monascus, Penicilliopsis, Penicillium, Phialomyces, Sclerocleista, Warcupiella, Xeromyces*), *Thermoascaceae* (*Byssochlamys/Paecilomyces, Thermoascus*) and the *Trichocomaceae* (*Rasamsonia, Sagenomella, Talaromyces, Thermomyces, Trichocoma*). *Penicillium* subgenus *Biverticillium* and *Talaromyces* were shown to form a monophyletic clade distinct from the other subgenera of *Penicillium*, with these names recombined as necessary into *Talaromyces* (Samson *et al*., 2011). The remaining *Penicillium* species formed a monophyletic clade together with species classified in *Eupenicillium, Eladia, Hemicarpenteles, Torulomyces, Thysanophora* and *Chromocleista*. These generic names were synonymised with *Penicillium*, while their species were given *Penicillium* names (Houbraken and Samson, 2011). The remaining three *Aspergillus* species, *A. paradoxus* (*Hemicarpenteles paradoxus*), *A. malodoratus* and *A.*

crystallinus, phylogenetically belonging in *Penicillium*, are transferred to *Penicillium* below in the Taxonomy section. To accommodate the morphological variation, the generic diagnosis of *Penicillium* was amended in Houbraken and Samson (2011). Most importantly, in comparison with the prevailing generic concept (Raper and Thom, 1949, Pitt, 1979), it now excludes the acerose phialides and usually symmetrically branched conidiophores of species now included in *Talaromyces*, and was expanded to include the conidiophores with solitary phialides of species in section *Torulomyces*, and the darkly pigmented stipes that formerly characterised the genus *Thysanophora*, which show secondary growth by means of the proliferation of an apical penicillus. For infrageneric classification, the genus was divided into two subgenera, *Aspergilloides* and *Penicillium*, and 25 sections.

Thom (1954) attempted to explicitly define species concepts used for *Penicillium*. He was the pioneer of standardized working techniques and emphasised that *Penicillium* taxonomy demands a consistent, logical approach. He demonstrated these tendencies himself by taking into account infraspecies variation when delineating species. To minimise infraspecies variation, the importance of standardized working techniques were again emphasized by Pitt (1979), Samson and Pitt (1985), Okuda (1994) and Okuda *et al*., (2000). Although this was relatively effective when dealing with freshly isolated or wild-type strains, comparing strains using only morphology requires experience and nuance, because of the degeneration of characters in old reference material and the large number of species in the genus. New techniques incorporated into taxonomic studies resulted in the physiological species concept (Ciegler and Pitt, 1970, Pitt, 1973, Frisvad, 1981, Frisvad and Filtenborg, 1983, Cruickshank and Pitt, 1987a,b, El-Banna *et al.*, 1987, Frisvad and Filtenborg, 1989, Paterson *et al.*, 1989), phylogenetic species concept, including Genealogical Concordance Phylogenetic Species Recognition (GCPSR) (LoBuglio *et al.*, 1993, Berbee *et al*., 1995, Boysen *et al.*, 1996, Geiser *et al.*, 1998, Skouboe *et al.*, 1999, O'Donnell *et al*., 1998, Peterson, 2000a, Taylor *et al*., 2000) and eventually led to the combined approach using morphological, extrolite and genetic data in a polyphasic species concept (Christensen *et al.*, 2000, Frisvad and Samson, 2004). In the modern taxonomy, however, sequence data and GCPSR carries more weight than morphology or extrolite data.

2.3 Morphological characterization of *Penicillium*

Morphology in the past has been central to the taxonomy of *Penicillium* and along with multigene phylogenetics and extrolite profiling comprises the polyphasic species concept adopted for *Penicillium*. Morphology is the physical design through which an organism functions in its environment. But it will change regularly due to environmental factors. Therefore strains characterized in one atmosphere might look diverse when their environmental and nutritional conditions are different. These effects can be minimized using uniform working techniques (Samson and Pitt, 1985, Okuda, 1994, Okuda *et al*., 2000).

2.3.a Macromorphology

Colony characters and diameters on specific media are important features for species identification. Czapek Yeast Autolysate agar (CYA) and Malt Extract agar (MEA, Oxoid) is recommended as standard media for *Penicillium*. Even though malt extract from Oxoid is recommended, many laboratories prefer Difco. It should be noted that two different MEA formulations are widely used in modern taxonomic studies. Blakeslee's MEA was historically widely used, but lately switched to a different formulation. Both are suitable for characterisation, but studies should state which MEA (Oxoid or Difco) and formulation was used. The following alternative media can be used for observing additional taxonomic characters: Czapek's agar (CZ), Yeast Extract Sucrose agar (YES), Oatmeal agar (OA), Creatine Sucrose agar (CREA), Dichloran 18 % Glycerol agar (DG18), Blakeslee's MEA and CYA with 5 % NaCl (CYAS). CZ was used in the taxonomic treatments of Raper and Thom (1949) and Ramírez (1982) and is chemically well defined.

2.3.b Micromorphology

Phialides and conidial characters of *Penicillium* are of great taxonomic importance. Conidiophore branching patterns were traditionally used in the classification of *Penicillium* (Thom, 1930, Raper and Thom, 1949, Pitt, 1979). Although these branching patterns do not correspond perfectly with the sections currently accepted for *Penicillium*, characterizing them accurately is still considered important. The conidiophores range from being simple (solitary phialides) to very complex patterns with multiple levels of branching resulting in overall symmetrical or asymmetrical patterns. Monoverticillate conidiophores have a terminal whorl of phialides and in

some species, the terminal cell of the conidiophore is slightly swollen or vesiculate; such species could be confused with diminutive *Aspergillus* conidiophores, but they have septa in the stipes unlike species of the latter genus. Divaricate conidiophores, previously also referred to as irregular (Pitt, 1979), are best described as having a simple to complex branching pattern with numerous subterminal branches formed, but where conidiophore parts are divergent. Biverticillate conidiophores have a whorl of three or more metulae between the end of the stipe and the phialides; the metulae may be of unequal or equal length, vary in their degree of divergence, are usually more or less cylindrical but can also be clavate or slightly vesiculate. Terverticillate conidiophores have another level of branching between the stipe and the metulae, often just a continuation of the stipe axis and one side branch, sometimes a true whorl of three or more branches. Quaterverticillate conidiophores are produced by only a few species, and have one extra level of branching beyond the terverticillate pattern. Terverticillate and quaterverticillate conidiophores tend to be conspicuously asymmetrical. In colonies of many species, especially as cultures begin to degenerate, there may be more than one branching pattern or intermediate forms, and it can be challenging to decide which pattern is typical or most developed. Other important microscopic characters include the wall texture/ornamentation of stipes and conidia, as well as dimensions, ornamentation and sometimes colours of all elements of the conidiophore. Wall textures are very sensitive to minute differences in media composition and aeration. For best observation of conidial ornamentation, differential interference contrast (Nomarski) is recommended if possible; ornamentation is sometimes most conspicuously visible in air pockets in the preparation.

2.4 Molecular characterization of *Penicillium*

During the 1990's, DNA sequencing became one of the most powerful tools for taxonomists, because it created the opportunity for inferring relationships between species without the need for standardizing culturing regimes and eliminated problems related to deteriorated cultures. It also created the opportunity for sequence based identifications. DNA barcoding was launched to make species identification of any eukaryotic organism possible for anybody, by using a standardized short DNA sequence and a curated reference database linked to authoritatively identified vouchers (Blaxter, 2003, Tautz *et al.*, 2003, Hebert *et al.*, 2003, Blaxter *et al.*, 2005,

DeSalle *et al.*, 2005, Ratnasingham and Hebert, 2007, Seifert *et al.*, 2007, Min and Hickey, 2007a,b, Schoch *et al.*, 2012). Recently, internal transcribed spacer rDNA (ITS) accepted as the universal barcode for fungi (Schoch *et al.*, 2012) due to availability of universal primers (Schoch *et al.*, 2012). Unfortunately, for *Penicillium* and many other genera of ascomycetes, the ITS is not good for distinguishing all closely related species (Schoch *et al.*, 2012).

Identification of *Penicillium* based on the Gene Bank sequence repository is difficult due to large numbers of incorrect sequences. This particular problem is addressed in a number of publications (Koljalg *et al.*, 2005, Santamaria *et al.*, 2012, Koljalg *et al.*, 2013, Schoch *et al.*, 2014). For *Penicillium,* the International Commission of *Penicillium* and *Aspergillus* (ICPA), in conjunction with the publication of an updated accepted species list presented below, decided to include Gene Bank accession numbers to reference barcode sequences for each species when available. Because of the limitations associated with ITS as a species marker in *Penicillium*, a secondary barcode or identification marker is often needed for identifying isolates to species level. The requirements for a secondary identification marker are clear. It should be easy to amplify, distinguish among closely related species and most importantly, the reference data set should be complete, meaning that there should be representative sequences for all species. It would be an added bonus if this marker is useful for phylogenetic studies, as it will by default become the gene most widely sequenced in future. Based on these criteria, β-tubulin (BenA) is the best option for a secondary identification marker for *Penicillium.* BenA does, however, have problems associated with it. Although not influencing BLAST identifications, alignments across a diverse genus like *Penicillium* is difficult and often contains a large proportion ambiguously aligned sites, which can make phylogenies difficult. Also, there is evidence for the amplification of BenA paralogous genes in *Aspergillus* (Peterson, 2008, Hubka and Kolarik, 2012) and *Talaromyces* (Peterson and Jurjevic, 2013) and it can thus be assumed that the same might be happening in some *Penicillium* species, although this has not been shown. Other possible secondary marker options include calmodulin (CaM) or the RNA polymerase II second largest subunit (RPB2) genes. Both these genes have similar discriminatory power as BenA. RPB2 has the added advantage of lacking introns in the amplicon, allowing robust and easy alignments when used for phylogenies, but it

is sometimes difficult to amplify and the database is incomplete. Similarly, we lack a complete CaM database. Thus, for routine identifications BenA is currently recommended, while for the description of new species, the use of ITS, BenA, CaM and RPB2 among the markers for multilocus sequence typing and GCPSR is suggested BenA can successfully be used for accurately identifying *Penicillium* species. However, as is the case for genes other than BenA, care should be taken in specific groups or situations. Infraspecies variation in BenA occurs in some *Penicillium* species as is observed in phylogenies published for *Penicillium* (Frisvad and Samson, 2004, Barreto *et al*., 2011, Peterson *et al*., 2011, Houbraken *et al*., 2011b,c, Rivera and Seifert, 2011, Rivera *et al*., 2012, Houbraken *et al*., 2012a, Visagie *et al*., 2013, 2014a, 2014b). This variation must be considered for identification purposes and especially when considering whether a strain might represent a new species. This means that in addition to the reference sequences of ex-type cultures sanctioned by ICPA, additional reference sequences are necessary to document sequence variation that differ from the ex-type. ICPA is currently working on populating such a validated database to capture infraspecies variation, but for the time being critical phylogenetic revisions of different sections should be referred to for reliable data. Alternatively, combining ITS, BenA, CaM and RPB2 from a suspected new species with sequences of the same markers from related species will aid in deciding whether a species is new or not, using GCPSR as explained in detail by Taylor *et al.*, (2000). This is in fact common run through in most studies describing and characterizing *Penicillium* species.

2.5 Secondary metabolites production of *Penicillium*

The first anamorphic genus characterized based on using secondary metabolite profiling was *Penicillium* (Smedsgaard and Frisvad, 1997). It was reported that all *Penicillium* species produced highly species-specific pattern of secondary metabolites (Frisvad and Samson, 2004; Frisvad *et al*., 2004a; Smedsgaard *et al.*, 2004). Volatile secondary metabolites could also be used to establish many species (Larsen and Frisvad, 1995a,b). Chemotaxonomic studies of *Talaromyces* (Frisvad *et al.*, 1990) and *Penicillium* subgenus *Furcatum* (Frisvad *et al.*, 2006) showed that the genus *Penicillium* can be classified based on secondary metabolites with morphology and molecular data.

Firstly, chemical profiling of fungi were based on paper chromatography and thin layer chromatography (TLC; Frisvad *et al.*, 1989; Lumbsch, 1998) but later it was concluded that these techniques are not reliable for species differentiation (Filtenborg and Frisvad, 1980, Filtenborg *et al*., 1983; Frisvad *et al.*, 1989).

HPLC was standardized for partial identification of many of the metabolites (Frisvad, 1987, 1989; Frisvad and Thrane, 1987, 1993). Capillary electrophoresis can also be used for separation of secondary metabolites (Nielsen *et al*., 1996), but it has not much use in chemotaxonomy. For volatile secondary metabolites, GC-mass spectrometry (GC-MS) has been used in *Penicillium* taxonomy (Larsen and Frisvad, 1994).

Extrolites are produced by the mycelium and sporulating structures of *Penicillium* species, and exudates, diffusible pigments, and reverse colours are also mixtures of secondary metabolites. Studies of extrolite profiles were very useful for unravelling some morphological species concepts into biologically meaningful segregate species before DNA sequencing provided similar possibilities. As an example, the *P. aurantiogriseum* complex, critical contaminants of grain, was divided first into species using extrolite profiles by Frisvad and Filtenborg (1983, 1989), delimitations that were subsequently supported by BenA sequencing (Seifert and Louis-Seize, 2000). Identification of unknown strains using extrolites is possible for well-equipped chemical laboratories. The best way of using extrolites as identification aids is to extract and then separate them by HPLC and then partially or fully identify as many of the secondary metabolites as possible, generally using mass spectroscopy based technology (Frisvad *et al*., 2008). The media used for identification, especially CYA and YES agars, are optimal for production of most major diagnostic extrolites in *Penicillium* after incubation for 7 days at 25℃ in darkness. Agar plugs are extracted with a mixture of dichloromethane, ethylacetate and methanol. The metabolites extracted can then be analysed using advanced separation and detection techniques, or example ultra high performance liquid chromatography with diode array detection and high resolution mass spectrometric detection (UHPLC-DAD-HRMS) (Kildgaard *et al*., 2014). However, simpler HPLC-diode array detection methods can also be used (Frisvad and Thrane, 1987, 1993). Laboratories with less sophisticated equipment can perform valuable confirmatory tests. Although Thin Layer Chromatography (TLC) is no longer considered state of

the art for chemical research, it is still a useful technique for detecting coloured or uncoloured extrolites that can be used to confirm the identification of a *Penicillium* strain. For example, *P. brevicompactum* consistently produces large amounts of the colourless mycophenolic acid, and this metabolite will make a green colour reaction with ferric chloride (Clutterbuck and Raistrick ,1933). Sometimes only a few extrolites are needed to confirm the identity of a *Penicillium* isolate. If an isolate produces griseofulvin and roquefortine C, it can only be *P. coprophilum, P. griseofulvum* or *P. sclerotigenum* and if the isolate also produces cyclopiazonic acid, it can only be *P. griseofulvum* (Frisvad and Samson, 2004). Identification based purely on secondary metabolites is not yet possible for all species of *Penicillium*, but future databases will be better developed and an optimal battery of media for secondary metabolite production may allow this method to be used for identification of all species in the future.

The present research work has been undertaken at the Division of Plant Pathology, ICAR- Indian Agricultural Research Institute, New Delhi. The main objectives of this study were morphological and molecular characterization of *Penicillium* species; and Chemo-profiling of *Penicillium* species and their taxonomical evaluation using morphological, molecular and secondary metabolic data.

3.1 Collection and isolation of *Penicillium* species

To carry out the above objectives, twenty samples were collected from different parts of the IARI fields. The isolation was done by serial dilution method (Durowade, 2009). One gram of soil sample was mixed in 10 ml of sterile distilled water to make spore suspension. One ml of spore suspension was spread onto Czapek Yeast Autolysate (CYA) Agar in a petri plate.

After 5 to 7 days of fungal growth, the petri plates were screened for *Penicillium* fungus. Further the *Penicillium* isolates were subjected to purification by using standard fungal isolation procedures. A total of 15 *Penicillium* isolates were obtained from all the samples collected. All the isolates were mentioned in the Table 1 along with source and place of collection.

Table 1. List of *Penicillium* isolates collected from IARI fields

Sl. No	Source/Host	Place of Collection	Assigned number for isolates
1	`Soil	IARI field	S-01
2	Soil	IARI field	S-02
3	Soil	IARI field	S-03
4	Soil	IARI field	S-07
5	Soil	IARI field	S-09
6	Soil	IARI field	S-11
7	Soil	IARI field	S-12
8	Soil	IARI field	S-13
9	Soil	IARI field	S-15
10	Soil	IARI field	S-16
11	Soil	IARI field	S-18
12	Soil	IARI field	S-19
13	Soil	IARI field	S-20
14	Soil	IARI field	S-21
15	Soil	IARI field	S-22

Twenty six more isolates of *Penicillium* were procured from Indian Type Culture Collection (ITCC), Division of Plant Pathology, ICAR- Indian Agricultural Research Institute, New Delhi (Table 2).

Table 2. List of *Penicillium* isolates added from ITCC

Sl. No	Source/Host	Place of Collection	ITCC No
1	Soil	West Bengal	1495
2	Soil	IARI, New DELHI	1601
3	Soil	Assam	2168
4	*Dolichos* sp	Nainital, Uttarakhand	2410
5	Rotten Orange	New Delhi	3122
6	Soil	Burdwan, West Bengal	3146
7	Soil	Darjeeling, West Bengal	3211
8	Soil	Lucknow, Uttar Pradesh	3212
9	Milk product	Karnal, Haryana	4254
19	Milk Product	Karnal, Haryana	4255
11	Poultry farm soil	Jammu, Jammu & Kashmir	4276
12	Soil	Jammu, Jammu & Kashmir	4482
13	Soil	Gwalior, Madhya Pradesh	4603
14	Soil	Hyderabad, Telangana	5080
15	Soil	Hyderabad, Telangana	5082
16	Soil	Abohar, Punjab	5235
17	Tendu Leaf	Chaphra, Bihar	5251
18	Soil	Calcutta, West Bengal	5285
19	Soil	Almora, Uttarakhand	5287
20	Gram Soil	Srinagar, Jammu & Kashmir	5292
21	Soil	IARI, New Delhi	5297
2	Soil	IARI, New Delhi	5298
23	Safed Musli	Madurai, Tamil Nadu	6093
24	Kinnow	Sriganganagar, Rajasthan	6171
25	Sea Food Processing Industry	Roorkee, Uttarakhand	6374
26	Tomato	IARI, New Delhi	6755

3.2 General laboratory work

3.2.1 Media used for study

Czapek Yeast Autolysate agar (CYA, Pitt 1979)

Components	Amount (g/l)
Czapek concentrate	10 ml
Sucrose	30 g
Yeast extract (Difco)	5 g
K_2HPO_4	1 g
Trace elements stock solution	1 ml
Agar	20 g
dH_2O	1000 ml

Mix well and autoclave at 121 °C for 15 min. pH 6.2 ± 0.2.

Trace element stock solution 100 ml

Components	Amounts (g/l)
$CuSO_4 .5H_20CUSO_4 .5H20$	0.5 g
$ZnSO_4 .7H2_OZnSO_4 .7H_2O$	0.1 g
dH_2OdH_2O	100 ml
Store at 4-10 ^oC	

3.2.2. Glassware

The glassware used in the present study were petri plates (90 mm), conical flasks, test tubes, beakers, slides and funnels etc. of Borosil and Corning brands. The glassware's were cleaned thoroughly with detergent solution and washed in running tap water. Cleaning solution (Potassium dichromate 75 g, conc. H_2SO4 500 ml and water 1000 ml) was also used for cleaning the glassware whenever required. Cleaned glass ware was finally autoclaved before use. The sterilized polystyrene petri plates were used for this study.

3.2.3. Equipment

Equipment used during the course of investigation included compound microscope with a mounted camera of Progres 2.7 version (Jenoptik make, USA), refrigerator, autoclave, hot air oven, B.O.D. incubator, laminar air flow, pestle and mortar, water bath, centrifugation unit, microwave oven, weighing balance, thermocycler, gel-electrophoresis unit, micro-pipettes, micro-tips, inoculation needle, scalpel, razor and glass cavity slides, glass rods, forceps, needles, parafilm tape, eppendorf tubes of 1.5 ml and 2 ml, whatman filter paper, aluminum foil, aluminum Thin Layer Chromatography plate, TLC chamber, uv light chamber, Rotary evaporator, Gas Chromatography Mass Spectrometry etc.

3.2.4. Sterilization

All types of glassware including petri test tubes, conical flasks and pipettes used in the present investigation were sterilized in a hot air oven at 180^{o}C for 2 h. Inoculation needle, forceps, etc. were flame sterilized during transfer of cultures. All liquid and semi-solid media were sterilized at 1.1 kg/cm^2 (121.6 ^{o}C) for 15 min in an autoclave. The inoculation (Laminar air flow) chamber was sterilized by switching on the UV lamps for at least 20-30 min and hands were sterilized with 70 % ethanol.

3.4. Identification of *Penicillium* species through morphological and molecular characterization

3.4.1. Morphological characterization of *Penicillium* isolates

3.4.1.1. Cultural characters

The cultural characteristics of *Penicillium* isolates grown on CYA were studied. Spore suspension of semi-solid solution containing 0.2% agar and 0.05% tween 80 was inoculated at the center of media in polystyrene petri plate. The plates were allowed for 7 days incubation at 25^{o}C. Then the observations were recorded on the following characteristics:

3.4.1.2. Macroscopic characters

Different species of *Penicillium* show different macroscopic characters such as colony colour, colony texture, Exudate, reverse colour, colony marginal colour and pigmentation.

3.4.1.3. Microscopic characters

Microscopic observations of isolates of *Penicillium* viz., conidiophore shape, phialide shape and structures composing of rami, metulae, stripe, shape and size of conidia were taken as a parameter of different species. Predominantly the isolates were identified based on the shape of conidia and phialide structure which are distinct from species to species. Slides were prepared with lactophenol and observed under the microscope. Slants containing 3-4 days old colony were picked by inoculating needle and fixed under the slide with cover slip. Then slide was slightly warm over the spirit lamp in order to melt the agar containing mycelium and taken care to prevent from boiling. The excess water was removed with the help of clean blotting paper and then slides were observed under 10X, 40X and 100X magnification. Observations on branching pattern and size and shape of the phialide were recorded. To obtain shape and size of conidia a drop of water was put over the slide and spores taken from the tube are mixed with water by inoculating needle. Since *Penicillium* is well known for vigorous spore production, spores were proper spread over the slide by using single drop of ethyl alcohol and fixed under the cover slip. The photo-micrographs of the above characters were taken using Progres 2.7 version (Jenoptik, USA) camera. Ten random observations were recorded while measuring the spore characteristics of different isolates.

3.4.2. Molecular characterization of *Penicillium* species using β-tubulin gene

3.4.2.1. DNA extraction

Genomic DNA was extracted from all the isolates of *Penicillium* using monosporic cultures by CTAB (Cetyl trimethyl Ammonium Bromide) method (Culling, 1992). 0.2g of mycelium mat of seven days old was collected from potato dextrose broth and grounded in sterilized pestle and mortar using liquid nitrogen and transferred to 1.5 ml eppendorf micro tubes. 600 µl of preheated (60^{o}C) 2 × CTAB extraction buffer (2 % (w/v) CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, pH 8.0) was added to the eppendorf micro tubes. The solution was incubated at 60^{o}C for one hour in water-bath with occasional gentle stirring. To this solution an equal volume of chloroform and isoamyl alcohol (24:1) was added and mixed thoroughly. The mixture was subsequently centrifuged at 10,000 rpm for 20 min at 24^{o}C. Aqueous phase was separated and transferred to a fresh tube. To this aqueous phase an equal volume of chloroform and isoamyl alcohol (24:1) was added and mixed thoroughly

and centrifuged at 10,000 rpm for 20 min at 24^{o}C. These steps were repeated 2 - 3 times till a clear aqueous phase was obtained. To this clear aqueous phase 0.6 volume of ice cold isopropanol and 0.1 volume of sodium acetate buffer (3M) was added and incubated at −20^{o}C for 30 minutes. DNA was precipitated by centrifuging at 10,000 rpm for 10 min at 4^{o}C. The precipitate was treated with 75 % ethanol and centrifuged at 10,000 rpm for 10 min at 4^{o}C. Aqueous phase was discarded and DNA was dried under a regular air flow for 20 min, re-suspended in 70 µl TE buffer and stored at −20^{o}C. The presence of DNA in the samples was further confirmed by separating them on 0.8 % agarose gel at 80 volts for 45 min using gel electrophoresis unit. The concentration of DNA was measured through spectrophotometrically using Nano drop 2000 spectrophotometer.

3.4.2.2. PCR amplification of β-tubulin gene

The identification of the purified isolates based on morphological characteristics was complemented with the sequencing of the β-tubulin gene. The amplification of β-tubulin gene was carried out using forward primers Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and reverse primer Bt2b(5'-ACC CTC AGTGTAGTGACCCTTGGC-3') (Glass and Donaldson, 1995). PCR reactions were carried out in 0.2 ml thin walled PCR tubes with a total reaction volume of 25 µl containing 12.5 µl of Dream Taq (2X) of Thermo Scientific make (master mix consisting of buffer, dNTP's, $MgCl_2$, Taq DNA polymerase at appropriate concentrations and pre mix of loading dye), 1 µl (10 Pmol/ µl) of each forward and reverse primers, 1 µl (100 ng/ µl) of DNA sample and nuclease free water. The PCR amplification conditions were initial denaturation at 94^{o}C for 5 min, 35 cycles of denaturation at 94^{o}C for 1 min, primer annealing at 61^{o}C for 1 min, primer extension at 72^{o}C for 2 min, followed by final primer extension at 72^{o}C for 5 min

3.4.2.3. Sequencing of the amplified β-tubulin gene

The amplified products were separated on 1.2 % agarose gel at 80 volts for 45 min using 1x TAE buffer (pH 8.0) containing ethidium bromide. The gels were photographed using gel documentation system. Amplicons of 500-550 bp were selected for sequencing the ITS region. For size selection a co-resolved 100 bp ladder was used. Sequencing of all the samples with distinct band was done through Eurofins Scientific, Bangalore.

3.4.2.4. Identification of *Penicillium* species through β-tubulin gene

Molecular identification of *Penicillium* spp. was done using nucleotide sequences of β-tubulin region through NCBI (National Centre for Biotechnology Information) BLAST (Basic Local Alignment Search Tool) (webpage: http://blast.ncbi.nlm.nih.gov) and the sequences were submitted to CBS-KNAW(Cenraalbureau voor Schimmelcultures fungal biodiversity centre) database.

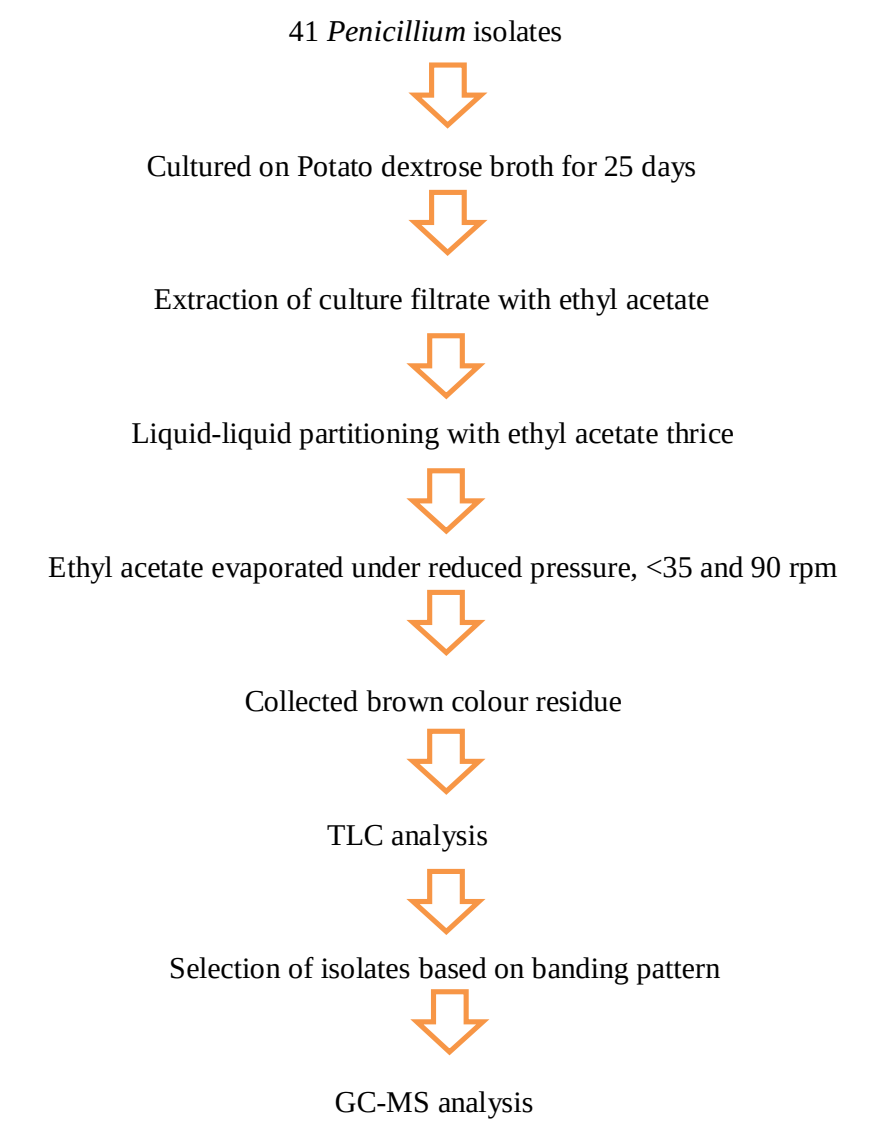
3.4.2.5. Phylogenetic analysis

Multiple sequence alignment of the β-tubulin gene sequences was performed using the Clustal W algorithm of MEGA 6.0 software. Phylogenetic tree was constructed using maximum parsimony (MP) analysis. Confidence value were assessed from 1000 bootstrap replicates of the original data.

3.4.3. Extraction of the secondary metabolites from *Penicilliium* isolates

Culture broth of forty one *Penicillium* isolates was extracted separately thrice with ethyl acetate (250 ml×3) in a separating flask. Extraction was carried out following liquid-liquid partitioning where ethyl acetate was added to the culture filtrate followed by agitated vigorously for 10 minutes. Then the separating flask kept undisturbed to separate the ethyl acetate layers. Ethyl acetate was taken out from the flask and passed through anhydrous sodium sulfate to remove traces of water, if any. After that extracts were combined and evaporated under *vacuum* at reduced pressure in rotary evaporator below 35^0C (90 rpm) to obtain brown coloured semi-solid crude ethyl acetate extract. Finally, weight of the dried residues were separately taken and kept under refrigerator for further use.

Extraction of secondary metabolites from *Penicillium* isolates



3.4.3.2. Thin Layer Chromatography (TLC)

TLC is an easy, cheap, rapid, and basic method to know the number of spots or organic compounds present in extract. TLC was carried out onto ready-made aluminum TLC plates where polar silica gel used as stationary phase. With a pencil, a thin mark was made at the bottom of the plate to load the sample spots in same line. Then, samples were loaded on their marked spots on the line in equal distances. The mobile phase (ethyl acetate and hexane, 99:1) was poured into the TLC chamber to a level few centimeters above the chamber bottom. A moistened filter paper in mobile phase is placed on the inner wall of the chamber to saturate with solvent vapour. Now, the plate prepared with sample spotting was placed in TLC chamber so that the

side of the plate with the sample line is facing towards the mobile phase. Then the chamber was closed with a lid. Keep the plate inside the chamber for sufficient time for the development of spots through the stationary phase. When the mobile phase moved approximately eighty percent of the plate, it was removed and allowed to dry. The sample spots were visualized under UV light chamber and further brown spots were seen with the iodine as visualizing agent. The banding pattern was converted into 0 and 1 manner (0-absent and 1-present) and dendogram was constructed by NTsys software.

3.4.3.3. Gas chromatography mass spectrometry (GC-MS) analysis of VOC

Nineteen ethyl acetate extracts extracted from various *Penicillium* isolates were analyzed by GC-MS to identify volatile organic compounds (VOC):

3.4.3.3.1 Sample preparation for GC-MS

After complete evaporation of solvents, the crude sample obtained was diluted with ethyl acetate solvent to prepare 10 ppm solution which was filtered out into a vial for further analysis

3.4.3.3.2. GC-MS condition:

GC-MS analysis was carried out using 7890A GC (Agilent Technologies, USA) equipped with a HP-5MS column (30 m × 0.25 mm;/0.25 µm, Agilent Co., USA) which was directly connected to a triple axis HED-EM 5975C mass spectrometer (Agilent Co., USA). The injection volume was 1 µl with flow mode in split control. The carrier gas flow was set at 1 mL min^{-1} helium. Helium (High purity, New Delhi, India) was used as carrier gas at a head pressure of 10 psi. GC-MS condition for volatile compounds is described as follows. The oven temperature was initially held at 40^0C for 1 min. Here after, the temperature was raised with a gradient of 2^0C min^{-1} until the temperature reached to 100^0C and held for 2 minutes. Again the temperature was raised with a gradient of 5^0C min-1upto 220^0C and held for 1 minute. Finally temperature raised up to 280^0C with an increment of 10^0C min-1.Total runtime was 64 minutes. The MS acquisition parameters were: ion source 180°C, electron ionization 70 eV, full scan mode (50–550 mass units), transfer line temperature 280°C, solvent delay 3 min, and E.M voltage 1376. The ionization energy was 70 eV with a scan time of 1 second and mass range of 50–550 AMU.

3.4.3.3.3. Identification of volatile compounds:

Compounds were identified by matching their mass spectra. NIST (National Institute of Standards and Technologies) Mass Spectra Library was used as a reference for identifying the essential components. Further components were identified from their main fragmentation patterns.

4.1. Collection and isolation of *Penicillium* species

Twenty soil samples collected from experimental field of ICAR-Indian Agricultural Research Institute (IARI), New Delhi were screened for the presence of *Penicillium* fungus through serial dilution method. Fifteen isolates were identified as *Penicillium* from the collected soil samples based on the macroscopic examination (colony colour, exudate of soluble pigment, reverse colouration, mycelial growth and colony texture) and microscopic observations (type and shape of penicillus; shape of phialides; conidial shape, size and pigmentation). Twenty six more isolates were procured from Indian Type Culture Collection, Division of Plant Pathology, ICAR-IARI, New Delhi.

4.2. Development of single spore cultures of *Penicillium*

Single spore isolations of all 41 cultures were made using 2% agar medium.

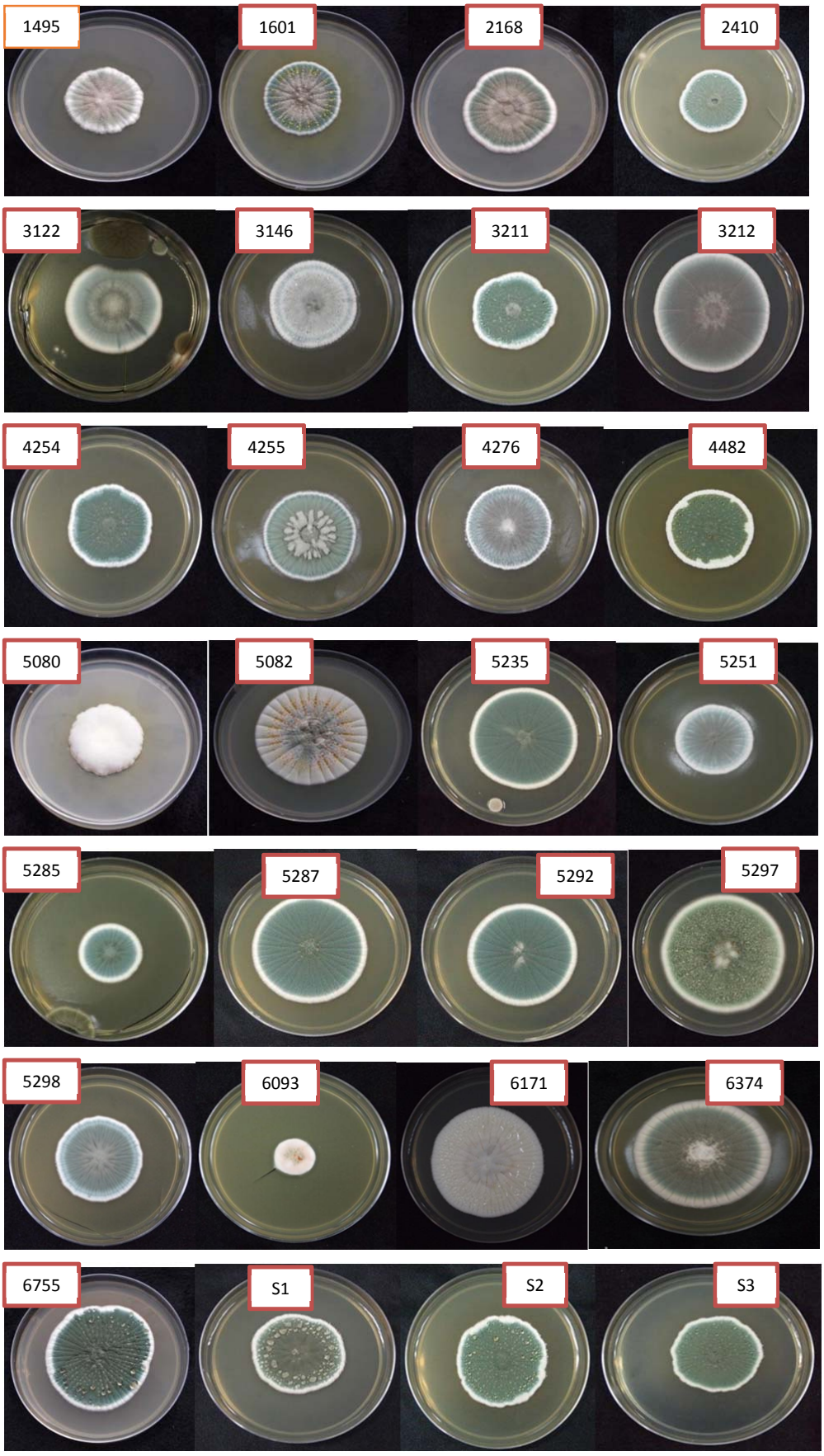
4.3. Morphological and molecular identification of *Penicillium* isolates

After single spore isolation, morphological and molecular studies were carried out for species identification based on morphological characters and β-tubulin gene sequence analysis.

4.3.1. Morphological characterization

4.3.1.1. Cultural characters

Colony characters and diameters on specific media are important features for species identification. Czapek Yeast Autolysate agar (CYA) was used for this study which is recommended as standard media for *Penicillium*. Cultural characters of 7 days old single spore isolates of 41 cultures incubated at 25^{o}C were used for study.There was wide variation in the radial growth of the isolates. Texture of the mycelium was found to be fine to velvety but no uniformity was observed among isolates. Different isolates produced different colony colours (Fig.1).


1495
1601
2168
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4254
4255
4276
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6374
6755
S1
S2
S3

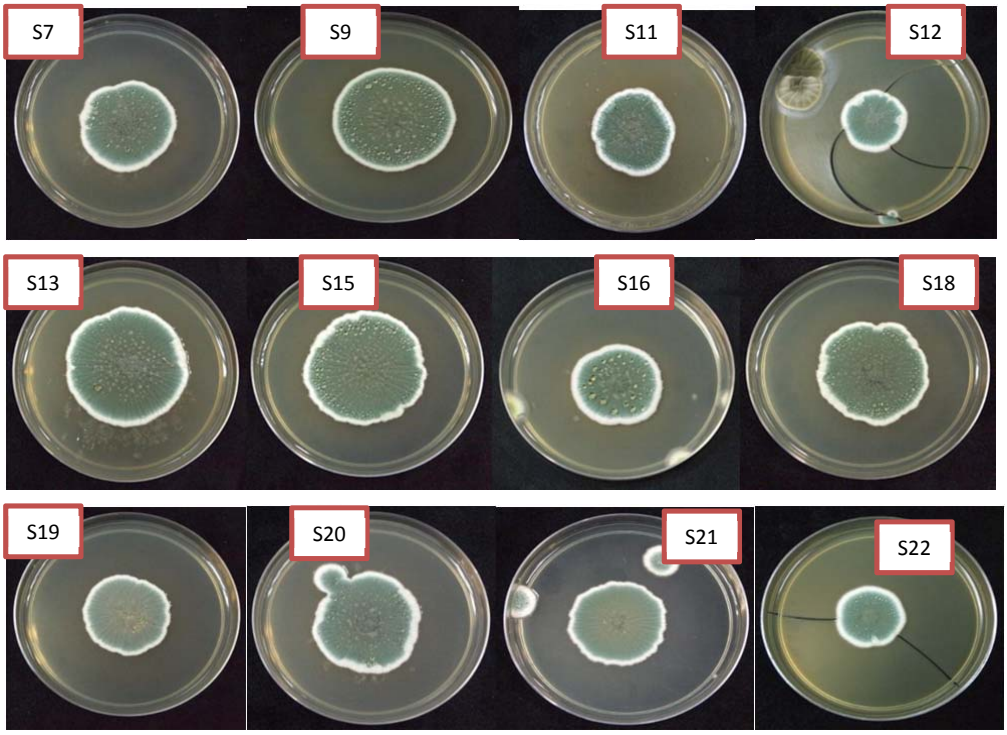

Fig. 1. Growth and sporulation of different *Penicillium* isolates

4.3.1.2. Microscopic characters

Based on the microscopic characters, all the 41 isolates were categorized into nine different groups (Table 3).

The Group- 1 isolates (2410, 3211, 4254, 4482, S1, S2, S3, S9, S11, S12, S13, S16, S18, S19, S21, S22, S20) were found with blue substrate and golden yellow reverse. Clear exudate was present. Soluble pigmentation was absent. Phialides were flask shaped with short collula and conidia were ellipsoidal, smooth walled with 2.8-3.2 x 3.3-3 µm size.

In Group-2 isolates (1495, 1601, 2168, 3122, 5080, 5082, S7), the following characters were observed. Substrate was bluish green to green in colour and reverse cream to yellow. Clear exudate was present. Yellow soluble pigmentation was present. Phialides were cylindrical with short collula and conidia were globose to subglobose or broadly ellipsoidal with 2.5-4 x 2.3-3.5 µm size

Group-3 has only one isolate (4255) which was dull green to grey green with blue green colony margin with yellowish brown reverse. Clear exudate was present. Pale brown soluble pigmentation was sometimes present. Phialides were cylindrical

tapering to a distinct collula and conidia were smooth walled, globose to subglobose with 3-4 x 3-4 µm size.

Among all the isolates, one isolate (5297) was put in Group 4 with distinct characters. Colony was greyish to dull green in colour with yellow to yellow brown reverse. Clear to brown exudate was present. Soluble pigmentation was absent. Phialides were flask shaped with long collula and conidia were sphaeroidal, pyriform to ellipsoidal with 2.2-3.0×2.0-2.5 µm size.

In Group-5, five isolates i.e., 3146, 3212, 4276, 5251, 6755 were found with the following characters. Bluish green to bluish grey substrate was present with cream to yellowish orange reverse. Clear exudate and brown pigmentation were present. Phialides were acerose with smooth, spherical to ellipsoidal conidia of 3.2-3.7 x 2.5-3.2 µm size.

One isolate (6374) was made into Group-6. Beigh green to dark green substrate was present with olive to brown reverse. Clear exudate was present. Soluble pigmentation was absent. Phialides were acerose with short collula. Conidia were large and ellipsoidal with 3.5 -5.0×2.5-4.0 µm size.

The isolates 5235, 5285, 5287, 5292, 5298, 6171were clustered into Group-7 with blue green colony substrate and yellow brown to red brown reverse. Clear exudate was present. Soluble pigmentation was absent. Phialides were flask shaped with short collula and conidia were smooth walled, globose to subglobose with 3-4 x 2.5- 3.5 µm size.

In Group-8 one isolate (4603) was found with greenish grey colony substrate and yellow to brown reverse. Exudate and soluble pigmentation was absent. Phialides were ampulliform and conidia were sub-globose to ellipsoidal with 2.5-3×2-3 µm size.

Two isolates i.e., 6093 and S15 in Group-9, showed greenish green to dull green substrate and orange to brown orange reverse. Red coloured exudate was present. Soluble pigmentation was absent. Phialides were acerose and conidia were globose to subglobose with 2–3 × 2–3 µm size.

4.3.1.4. *Penicillium* species confirmation

With the available literature (Pitt, 1979, Frisvad and Samson, 2004, Visagie *et al.*, 2014, Yilmaz *et al.*, 2014) these different groups of *Penicillium* isolates were confirmed into different species as given in Table 4.

4.3.2. Molecular identification of *Penicillium* isolates based on β-tubulin gene sequence analysis

PCR amplification of β-tubulin (BenA) gene of all the forty one isolates of *Penicillium* was done using the Bt2a and Bt2b primers. The amplified products were separated and sequenced. The sequence length of β-tubulin gene was 500-550bp. Gel photograph of 41 amplified products is shown in Fig. 2. Molecular identification of *Penicillium* spp. was done through NCBI BLAST(webpage: http://blast.ncbi.nlm.nih.gov) and CBS-KNAW database using β-tubulin gene sequences with sequence similarity of 93-100% (Table 5).

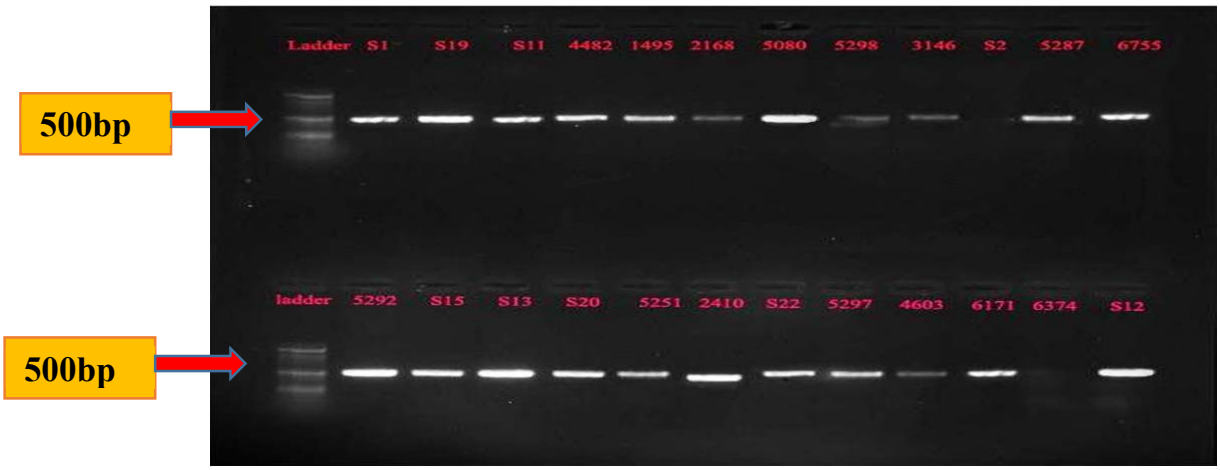

Fig.2. β-tubulin gene amplification of *Penicillim* spp. (Lane 1-41) with 100bp ladder

Table 3.Summary of morphological features for distinguishing 41 isolates of 9 *Peniciilum* spp.

Species Name	Radial growth rate after 7 days (in mm) CYA		Macro-morphological characterization on CYA media				Micro-morphological characters			Isolate Nos.
	Range	Mean	Substrate	Reverse	Exudate	Pigmentation	Phialide	Conidia shape	Conidia size (µm)	
Group-1	23.5-28	26	Blue	Golden yellow	Present, clear	Absent	Flask shape with short collula	Ellipsoidal smooth walled	2.8-3.2 x 3.3-3.8	2410, 3211, 4254, 4482, S1, S2, S3, S9, S11, S12, S13, S16, S18, S19, S21, S22, S20
Group-2	27.5-32	29.87	Blue green to green	Yellow brown to rarely brown	Present, clear	Yellow	Cylindrical with short broad collula	Globose to subglobose to broadly ellipsoidal	2.5-4 x 2.3-3.5	1495, 1601, 2168, 3122, 5080, 5082, S7
Group-3	28-34	32.5	Dull green to	Yellowish brown	Clear	Pale brown	Cylindrical tapering to a	Smooth walled	3-4 x 3-4	4255

			grey green or blue green colony margin				distinct collula	globose to subglobose		
Group-4	43-47	45.5	Greyish to dull green	Yellow to yellow brown	Clear to brown	Absent to Reddish brown	Flask shape with long collula	Sphaeroidal, pyriform to ellipsoidal	2.2-3.0×2.0-2.5	5297
Group-5	32-35.5	33.5	Bluish green to bluish grey	Cream to yellowish orange	Present, clear	Brown	Acerose	Spherical to ellipsoidal smooth	3.2-3.7 x 2.5-3.2	3146, 3212, 4276, 5251, 6755
Group-6	27-30	28.25	Beigh green to dark green	Olive to brown	Clear	Absent	Acerose with short collula	Large ellipsoidal	3.5 - 5.0×2.5-4.0	6374

Group-7	27.5-32	30	Blue green	Yellow brown to red brown	Present, clear	Absent	Flask shape with short collula	Smooth walled globose to subglobose	3-4 x 2.5-3.5	5235, 5285, 5287, 5292, 5298, 6171
Group-8	26.25-31.5	28.87	Greenish grey	Yellow to brown	Absent	Absent	Ampuliform	Sub-globose to ellipsoidal	2.5-3×2-3	4603
Group-9	19.5-24	22	Greenish green to dull green	Orange to brown orange	Red	Absent	Acerose	Globose to subglobose	2–3 × 2–3	6093, S15

Table 4. Confirmation of *Penicillium* species based on the identification key from (Pitt, 1979, Frisvad and Samson, 2004, Visagie *et al.*, 2014, Yilmaz *et al.*, 2014)

Group	*Penicillium* isolates	Identified as
Group-1	2410, 3211, 4254, 4482, S1, S2, S3, S9, S11, S12, S13, S16, S18, S19, S20, S21 and S22	*P. aethiopicum*
Group-2	1495, 2168, 3122, 5080 and 5082	*P. chrysogenum*
Group-3	4255	*P. crustosum*
Group-4	5297	*P. janthinellum*
Group-5	3146, 3212, 4276, 5251 and 6755	*P. mononematosum*
Group-6	6374	*P. oxalicum*
Group-7	5235, 5285, 5287, 5292, 5298 and 6171	*P. polonicum*
Group-8	4603	*P. singorense*
Group-9	6093 and S15	*Talaromyces pinophilus* *Syn. Penicillium pinophilum*

4.3.3. Phylogenetic analysis of β-tubulin gene

The dendrogram (Fig. 3) showed that the nine species of *Penicillium* were grouped into seven clusters. The isolates (2410, 3211, 4254, 4482, S1, S2, S3, S9, S11, S12, S13, S16, S18, S19, S20, S21 and S22) of *P. aethiopicum* were grouped in Cluster 1. In cluster 2 the isolates (3146, 3212, 4276, 5251 and 6755) of *P. mononematosum*. All the isolates of *P. chrysogenum* (1495, 2168, 3122, 5080 and 5082) were grouped in cluster 3. Six isolates (5235, 5285, 5287, 5292, 5298 and 6171) of *P. polonicum*were grouped in cluster 4 with one isolate of *P. crustosum* (4255). In Cluster 5, *P. janthinellum* (5297) and *P. singorense* (4603) were grouped together. Cluster 6 was with two isolates of *Talaromyces pinophilus*(6093 and S15**).** *P. oxalicum* (6374) was present alone in Cluster 7. Therefore, the isolates of *Penicillium* were grouped in homogenous manner.

4.3.4. Correlation of morphological and β-tubulin gene based identification of *Penicillium* species

All the forty one isolates were compared for morphological and molecular identity. Morphological identification of all the isolates was matched with molecular identification (Table 5).

4.4. Chemo-profiling of *Penicillium* isolates

4.4.1. Thin Layer chromatography(TLC) analysis:

Forty one *Penicillium* isolates were subjected to analyze TLC by using standard solvent solution ethyl acetate and hexane in the ratio of 99:1. Based on the banding patterns (Fig. 4), dendogram was constructed using NTsys software (Fig. 5). Representative isolates (19) from each cluster were selected for further analysis.

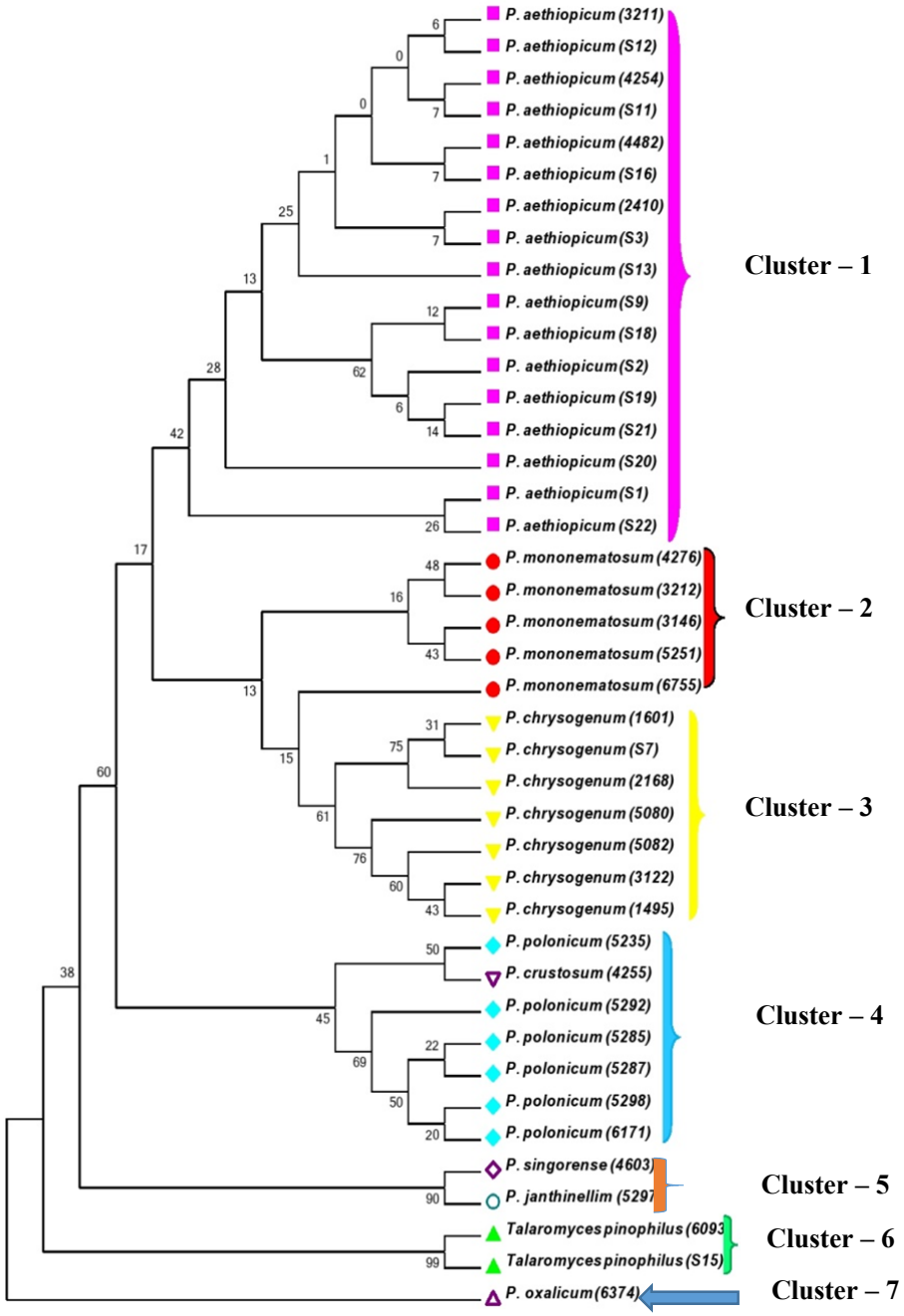

Fig. 3. Maximum parsimonious phylogenetic tree based on β-tubulin gene of different *Penicillium* isolates segregating into 9 *Penicillium* species

Table.5.Correlation of morphological and β-tubulin gene based identification of *Penicillium* species

Sl. No.	Isolates no.	Morphological identification	β-tubulin sequence based identification	Similarity with NCBI sequences (%)
1	2410	*P. aethiopicum*	*P. aethiopicum*	99
2	3211	*P. aethiopicum*	*P. aethiopicum*	99
3	4254	*P. aethiopicum*	*P. aethiopicum*	99
4	4482	*P. aethiopicum*	*P. aethiopicum*	99
5	S1	*P. aethiopicum*	*P. aethiopicum*	99
6	S2	*P. aethiopicum*	*P. aethiopicum*	99
7	S3	*P. aethiopicum*	*P. aethiopicum*	99
8	S9	*P. aethiopicum*	*P. aethiopicum*	99
9	S11	*P. aethiopicum*	*P. aethiopicum*	99
10	S12	*P. aethiopicum*	*P. aethiopicum*	100
11	S13	*P. aethiopicum*	*P. aethiopicum*	100
12	S16	*P. aethiopicum*	*P. aethiopicum*	99
13	S18	*P. aethiopicum*	*P. aethiopicum*	100
14	S19	*P. aethiopicum*	*P. aethiopicum*	99
15	S20	*P. aethiopicum*	*P. aethiopicum*	94
16	S21	*P. aethiopicum*	*P. aethiopicum*	99
17	S22	*P. aethiopicum*	*P. aethiopicum*	99
18	1495	*P. chrysogenum*	*P. chrysogenum*	99
19	1601	*P. chrysogenum*	*P. chrysogenum*	99
20	2168	*P. chrysogenum*	*P. chrysogenum*	99
21	3122	*P. chrysogenum*	*P. chrysogenum*	98
22	5080	*P. chrysogenum*	*P. chrysogenum*	98
23	5082	*P. chrysogenum*	*P. chrysogenum*	98
24	S7	*P. chrysogenum*	*P. chrysogenum*	98
25	4255	*P. crustosum*	*P. crustosum*	97
26	5297	*P. janthinellum*	*P. janthinellum*	97
27	3146	*P. mononematosum*	*P. mononematosum*	97
28	3212	*P. mononematosum*	*P. mononematosum*	94
29	4276	*P. mononematosum*	*P. mononematosum*	93
30	5251	*P. mononematosum*	*P. mononematosum*	98
31	6755	*P. mononematosum*	*P. mononematosum*	99
32	6374	*P. oxalicum*	*P. oxalicum*	93
33	5235	*P. polonicum*	*P. polonicum*	98
34	5285	*P. polonicum*	*P. polonicum*	99
35	5287	*P. polonicum*	*P. polonicum*	99
36	5292	*P. polonicum*	*P. polonicum*	99
37	5298	*P. polonicum*	*P. polonicum*	99
38	6171	*P. polonicum*	*P. polonicum*	99
39	4603	*P. singorense*	*P. singorense*	97
40	6093	*Talaromyces pinophilus*	*Talaromyces pinophilus*	98
41	S15	*Talaromyces pinophilus*	*Talaromyces pinophilus*	99

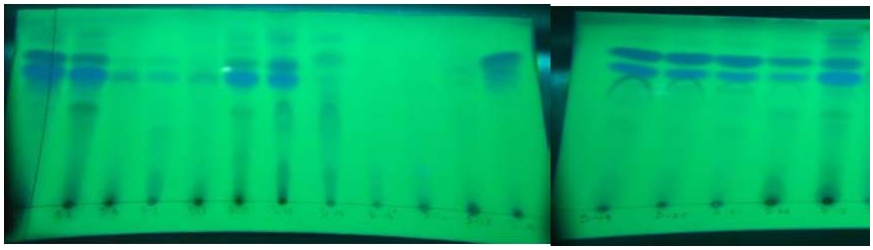

Fig 4: Banding pattern of *Penicillium* isolates using TLC under UV light

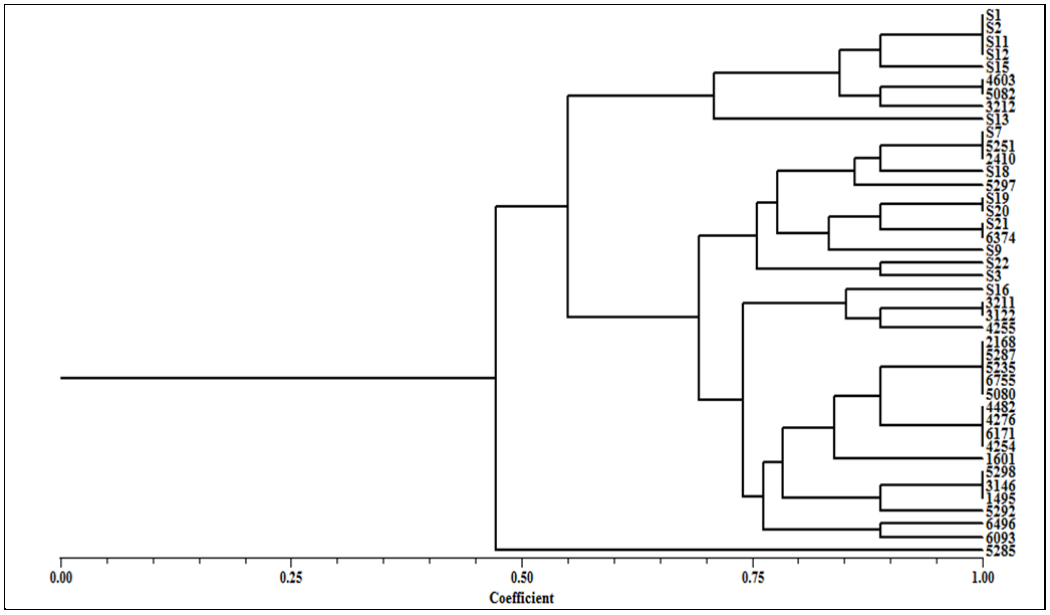

Fig 5: Clustering of 41 *Penicillium* isolates based on TLC banding pattern using NTsys software

4.4.2. Gas chromatography mass spectrometry (GC-MS) analysis:

Total nineteen isolates were analyzed for the production of volatiles compounds.

4.4.2.1. Chemical composition of (VOC) of *Penicillium* species:

GC-MS chromatogram exhibited few similar peaks in all the eight isolates of *Penicillium aethiopicum*. Two antibacterial antibiotic namely griseofulvin and dechlorogriseofulvin was identified in almost all isolates of *Penicillium aethiopicum* except in S3 and S16 due to very low concentration of these compounds in S3 and S16, therefore detection could not possible. Among the two known antibiotics dechlorogriseofulvin (12.08-55.68%) was identified as major constituents in all most

all the isolates, followed by griseofulvin (39.64-80.11%). 1,2-Benzenedicarboxylic acid was identified in all the isolates except S3 isolate and maximum abundance of this compound was observed in S16 isolates (77.97%). Similarly 9,12-octadecadienoic acid was also detected in almost all isolates, among them S3 isolate showed the maximum area percentage. Interestingly, hexadecane one of the hydrocarbon was also detected in various isolates of *Peniciliium aethiopicum* (Fig.6, Table 6).

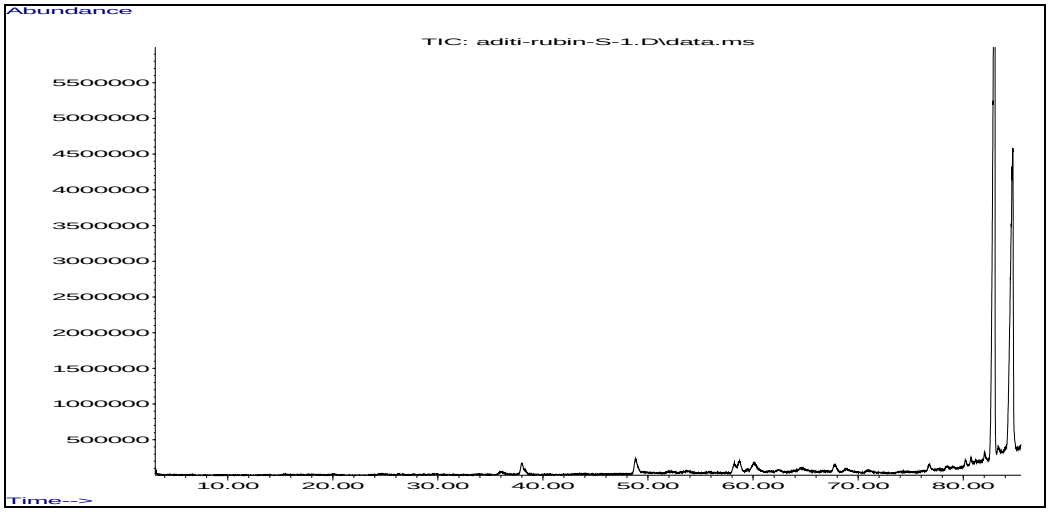

Fig.6. Total ion chromatogram (TIC) of GC-MS analysis of ethyl acetate extract of the *Penicillium aethiopicum*

Table 6. List of chemicals present in *Penicillium aethiopicum* isolates

Sl. no	Chemical name	Isolates (area%)							
		S1	**S19**	**S21**	**S22**	**S13**	**S16**	**S18**	**S3**
1	**Griseofulvin**	35.51	24.40	12.08	19.35	55.68	-	14.91	-
2	**Dechlorogriseofulvin**	54.14	67.35	80.11	45.26	39.64	-	78.62	-
3	9,12-Octadecadienoic acid	0.08	0.08	-	0.19	-	1.51	0.01	30.33
4	1,2-Benzenedicarboxylic acid	1.50	1.03	1.87	5.12	0.25	77.97	1.33	-
5	Hexadecane	0.25		0.58	0.96	-	-	-	-
	Total	91.48	92.86	94.64	70.88	95.57	79.48	1.33	

Similarly, three isolates of *Penicillium chrysogenum* were extracted individually with ethyl acetate and analysed by GC-MS. Few similar peaks were observed in GC-MS chromatogram to all the isolates of *P. chrysogenum.*Docosonal was identified as common constituents among the isolates. Another four compounds hexadecane, octadecene, eicosene and pentadecyl-heptafluorobutyrate was identified in 3122 and 5080 isolates. Cyclohexadecane and octadecanoic acid was found to be present in 5080 and S7 isolates. Surprisingly griseofulvin (10.44%) was observed as one of the major constituentin in S7 isolate (Fig.7, Table7).

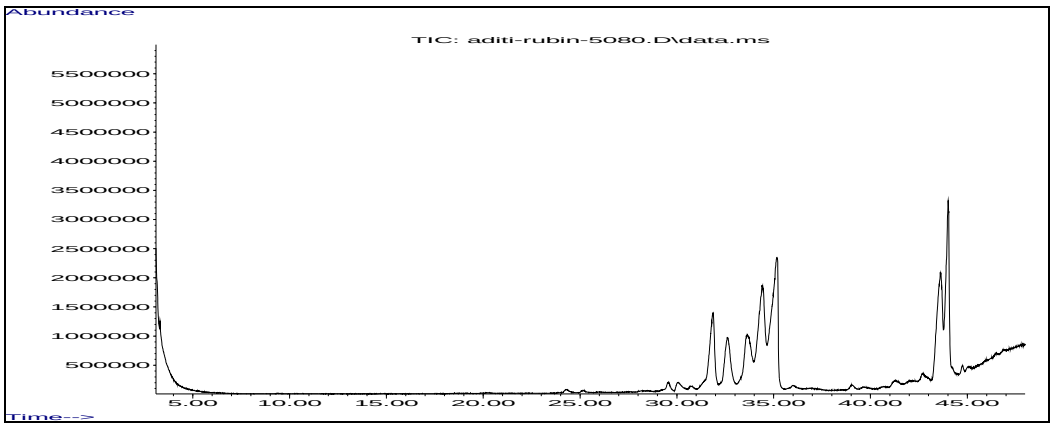

Fig 7.Total ion chromatogram (TIC) of GC-MS analysis of ethyl acetate extract of the *Penicillium chrysogenum*

Table7. List of chemicals present in *Penicillium chrysogenum*isolates

SL.NO	Chemical name	Isolates (area %)		
		3122	**5080**	**S7**
1	Hexadecene	1.99	0.11	-
2	Octadecene	4.88	0.77	-
3	Eicosene	7.74	0.02	-
4	Hexadecanoic acid	1.69	-	12.64
5	Docosanol	1.11	0.28	1.38
6	Pentadecyl-heptafluorobutyrate	0.52	0.06	-
7	Cyclohexadecane	-	0.20	0.57
8	Octadecanoic acid	-	0.12	2.58
9	**Griseofulvin**	-	-	10.44
	Total	17.93	1.56	17.17

Ethyl acetate extract of *Penicillium crustosum* was subjected to GC-MS analysis which exhibited major peaks correspond to pyrrolo pyrazine-1,4-dione (59.22%) and chloro-2-phenoxyethylene (3.22%). Twelve volatile organic compounds

constituents representing 64.48% of the total composition were identified in the ethyl acetate extract. Other compounds were 1,2-benzenedicarboxylic acid (0.72%), hexacosene (0.53%), docosene (0.41%) and octadecene (0.21%) were also identified. Pyrrolo pyrazine-1,4-dione (59.22%) was found to be the most abundant (Fig. 8, Table 8).

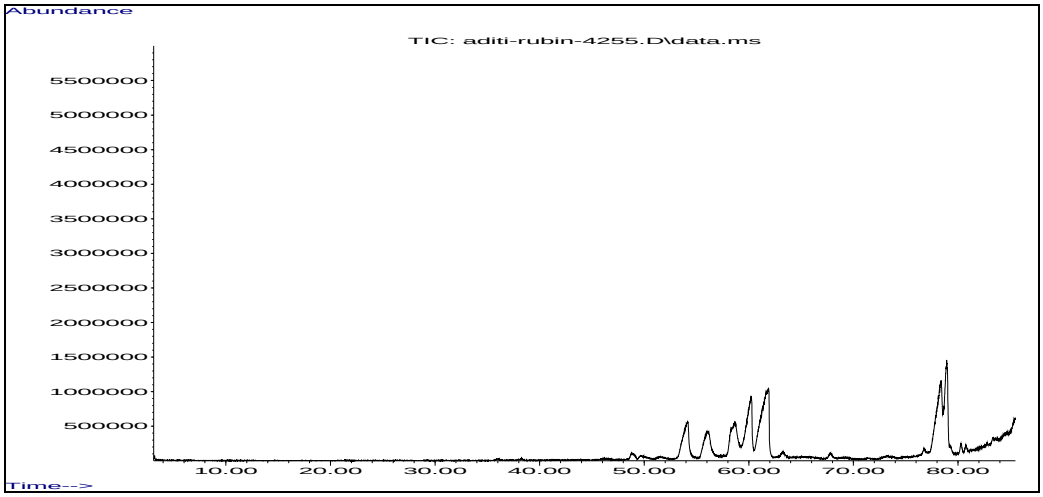

Fig 8.Total ion chromatogram (TIC) of GC-MS analysis of ethyl acetate extract of the *Penicillium crustosum*

Table 8. List of chemicals present in *Penicillium crustosum* isolate

S.N	COMPOUND NAME	AREA%
1	9,12-Octadecadienoic acid	0.08
2	Hexadecane	0.01
3	Octadecene	0.21
4	E-15-Heptadecenal	0.05
5	Pyrrolo-pyrazine-1,4-dione	59.22
6	Chloro-2-phenoxyethylene	3.22
7	3-Methyl-4-nitro-benzenamine	0.14
8	Octadecyltrifluoroacetate	0.03
9	Piperazinedione	0.06
10	Docosene	0.41
11	1,2-Benzenedicarboxylic acid	0.72
12	Hexacosene	0.53
	Total	64.48

GC-MS analysis of another ethyl acetate extract of *Penicillium janthinellum* exhibited mainly pyrrolo-pyrazine-1,4-dione (47.86%) and 1,2-benzenedicarboxylic acid (12.31%) as major constituents. Total twelve volatile organic compounds

constituents representing 63.15% of the total composition were found to be present in the ethyl acetate extract. Hexadecane (0.98%), 5-isopropyl-piperazine (0.55%) and eicosene (0.48%) were also identified. Pyrrolo-pyrazine-1,4-dione (47.86%) was the major constituent (Fig. 9, Table 9).

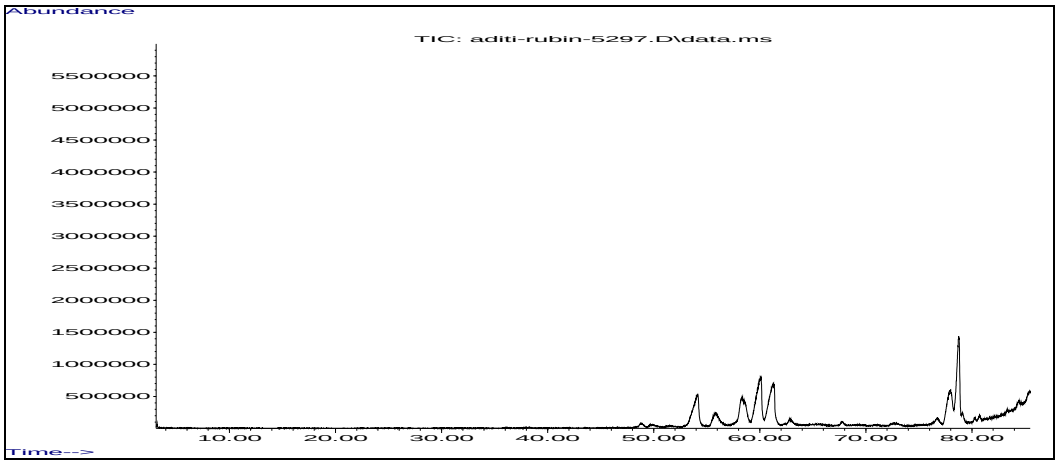

Fig 9.Total ion chromatogram (TIC) of GC-MS analysis of ethyl acetate extract of the *Penicillium janthinellum*

Table 9. List of chemicals present in *Penicillium janthinellum* isolate

S.N	COMPOUND NAME	AREA%
1	Hexadecene	0.98
2	Octadecane	0.01
3	Pyrrolo-pyrazine-1,4-dione	47.86
4	2H-Pyrrol-2-one	0.02
5	1,2-Benzenedicarboxylic acid	12.31
6	Nonadecyl-trifluoroacetate	0.26
7	Piperazinedione	0.03
8	5-Isopropyl-piperazine	0.55
9	Eicosene	0.48
10	1,2-Benzenedicarboxylic acid	0.45
11	Cyclotetracosane	0.05
12	17-Pentatriacontene	0.15
	Total	63.15

GC-MS analysis of ethyl acetate extract of *Penicillium mononematosum* was show the major peaks correspond to pyrrolo-pyrazine-1,4-dione (38.52%), 1-octadecene (1.46%) and 1-docosene (1.44%). Fifteen volatile organic compound constituent representing 45.23% of the total composition was characterized.

Tetradecen-1-trifluoroacete (0.80%), 9,12-octadecadienoic acid ethyl ester (0.57%), 1-hexadecene (0.54%), and β-eudesmol (0.51%) were also identified as major composition (Fig.10, Table10).

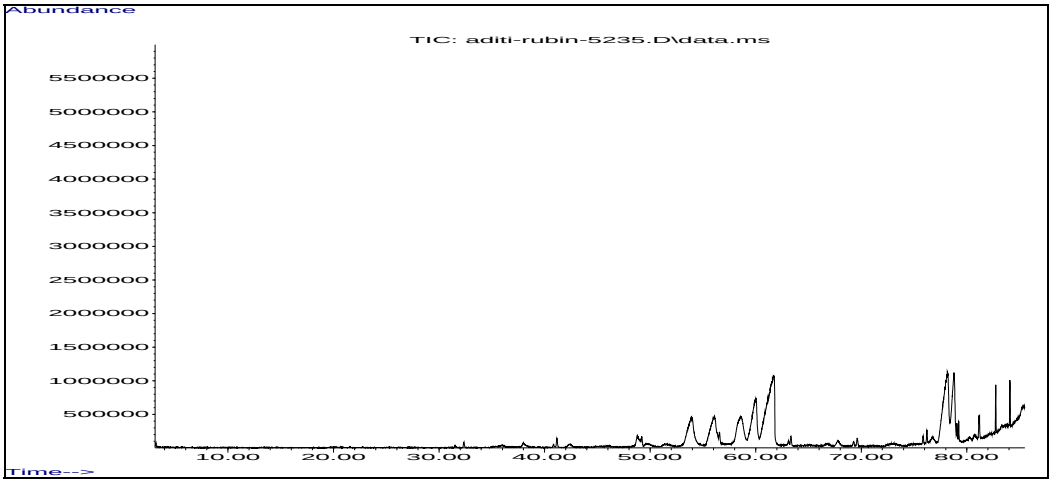

Fig 10.Total ion chromatogram (TIC) of GC-MS analysis of ethyl acetate extract of the *Penicillium mononematosum*

Table 10. List of chemicals present in *Penicillium mononematosum* isolate

S.N	COMPOUND NAME	AREA%
1	9,17-octadecadienal	0.09
2	Tridecene	0.13
3	Hexadecene	0.54
4	β-Eudesmol	0.51
5	octadecene	1.46
6	Pyrrolo-pyrazine-1,4-dione	38.52
7	9,12-Octadecadienoic acid-ethyl ester	0.57
8	Tetradecen-1-ol trifluoroacete	0.80
9	Docosene	1.44
10	Piperazinedione	0.22
11	Octacosyl-acetate	0.05
12	Octacosyl-heptafluorobutyrate	0.43
13	Nonadecene	0.09
14	17-Pentatriacontene	0.34
15	Oleic acid- propyl ester	0.04
	Total	45.23

Three isolates of *Penicillium polonicum* were individually extracted with ethyl acetate and further subjected to analyzed for chemical profiling of volatile organic constituents by GC-MS. Several compounds were found to be present in

given isolates of *P. polonicum*. Among them, hydrocarbon compound hexadecane exhibited sharp peak in all three isolates. Another two compounds, docosene and pyrrolo-pyrazine-1,4-dione were also identified in 5235 and 6171 isolates. Pyrrolo-pyrazine-1,4-dione was identified as a major organic constituents (38.50-38.52%) in the *P. polonicum*. On the other hand, 9,12- octadecadienoic acid ethyl ester was found in 5292 (4.21%) and 5235(0.57%) isolates (Fig.11,Table 11).

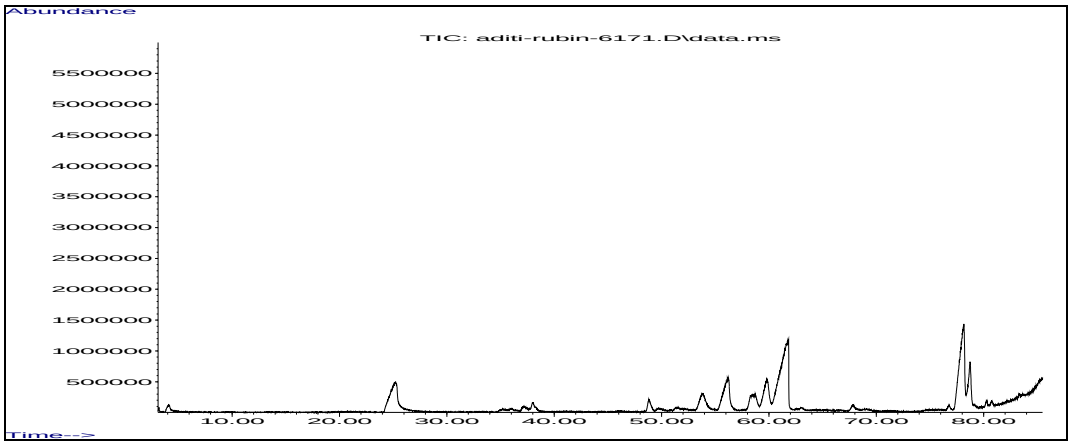

Fig 11.Total ion chromatogram (TIC) of GC-MS analysis of ethyl acetate extract of the *Penicillium polonicum*

Table 11. List of chemicals present in *Penicillium polonicum* isolates

Sl. no	Chemical name	Isolates (area%)		
		5292	**5235**	**6171**
1	Hexadecene	1.91	0.54	0.68
2	Eicosene	1.53	-	0.55
3	Docosene	-	1.44	0.20
4	Pyrrolo-pyrazine-1,4-dione	-	38.52	38.50
5	9,12-Octadecadienoic acid-ethyl ester	4.21	0.57	-
	Total	7.65	41.07	39.93

GC-MS analysis of ethyl acetate extract of *Talaromyces pinophilus* exhibited sharp peaks correspond to griseofulvin (66.62%), 1-octadecene (2.10%), 2,5-di-butyl-phenol (1.96%), eicosene (1.90%), docosene (1.87%), hexadecene (1.54%), and 1,2-benzenedicarboxylic acid (1.31%). Total twelve volatile organic compounds representing 78.78% of the total composition were identified in the ethyl acetate extract. Griseofulvin (66.62%) was identified as the major composition followed by octadecene (2.10%), 2,5-di-butyl-phenol (1.96%), eicosene (1.90%), docosene

(1.87%) and hexadecene (1.54%). Other identified minor composition were1,2-benzenedicarboxylic acid (1.31%), hexadecanoic acid (0.59%), 1-hexacosene (0.49%), methyl 10,12-octadecadinoate (0.23%), 9,12-octadecadienoic acid (0.10%), benzofuran-2-one (0.07%) (Fig.12, Table 12).

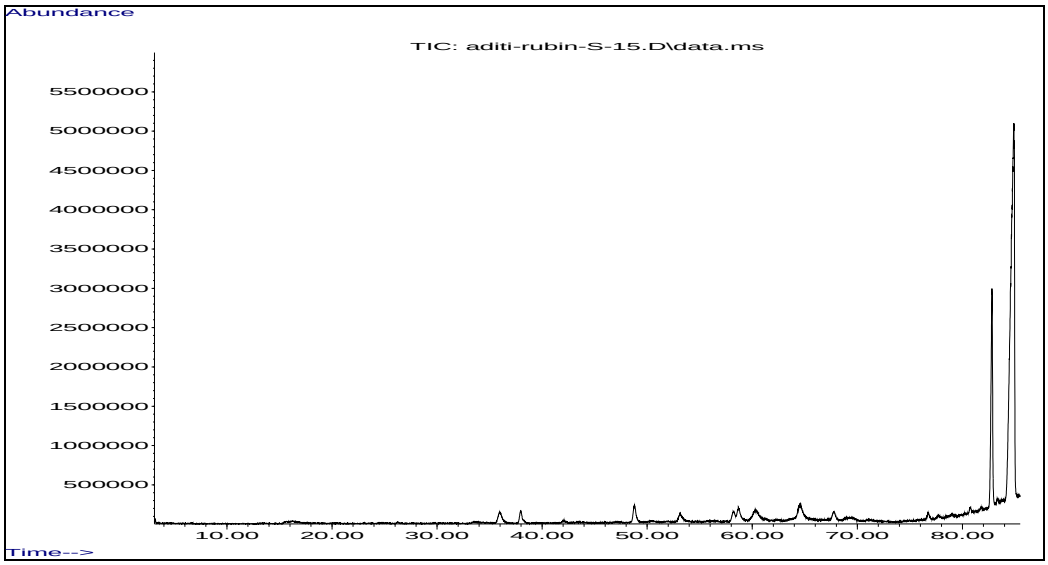

Fig 12.Total ion chromatogram (TIC) of GC-MS analysis of ethyl acetate extract of the *Talaromyces pinophilus*

Table 12. List of chemicals present in *Talaromyces pinophilus* isolate

S.N	COMPOUND NAME	AREA%
1	9,12-Octadecadienoic acid	0.10
2	Di-Butyl-Phenol	1.96
3	Hexadecene	1.54
4	Benzofuran-2-one	0.07
5	Octadecene	2.10
6	1,2-Benzenedicarboxylic acid	1.31
7	Eicosene	1.90
8	Hexadecanoic acid	0.59
9	Docosene	1.87
10	Hexacosene	0.49
11	Methyl -10,12-octadecadinoate	0.23
12	**Griseofulvin**	66.62
	Total	78.78

4.5. Polyphasic Taxonomy confirmed through morphology, molecular and chemo profiling data:

4.5.1. *Penicillium aethiopicum* (Fig.13):

In *Penicillium* subgenus *Penicillium* section *Chrysogena* series *Aethiopica*

Diagnostic features: Griseofulvin, viridicatumtoxin, tryptoquialanins, geosmin, ellipsoidal smooth-walled conidia, markedly sulcate colonies with a golden yellow reverse, growth at 37°C.

Description:

Conidia: Smooth-walled, ellipsoidal, 2.8-3.2 x 3.3-3.8 µm, in long columns

Phialides: 7-9 µm, short collula

Metulae: 12-17 µm

Rami: 15-25 µm

Stipes: 200-350 µm, smooth to rough-walled

Synnemata or fasciculation: Weakly fasciculate

Sclerotia: None

Colony texture: Sulcate on CYA

Conidiumcolour CYA: Dull green.

Exudate droplets on CYA: Copious, clear

Reverse colour on CYA: Golden yellow

Diffusible colour: Occasional; pale orange

Ehrlich reaction: No reaction

Odour and volatile metabolites: Griseofulvin, Dechlorogriseofulvin, 9,12-Octadecadienoic acid, 1,2-Benzenedicarboxylic acid and Hexadecane.

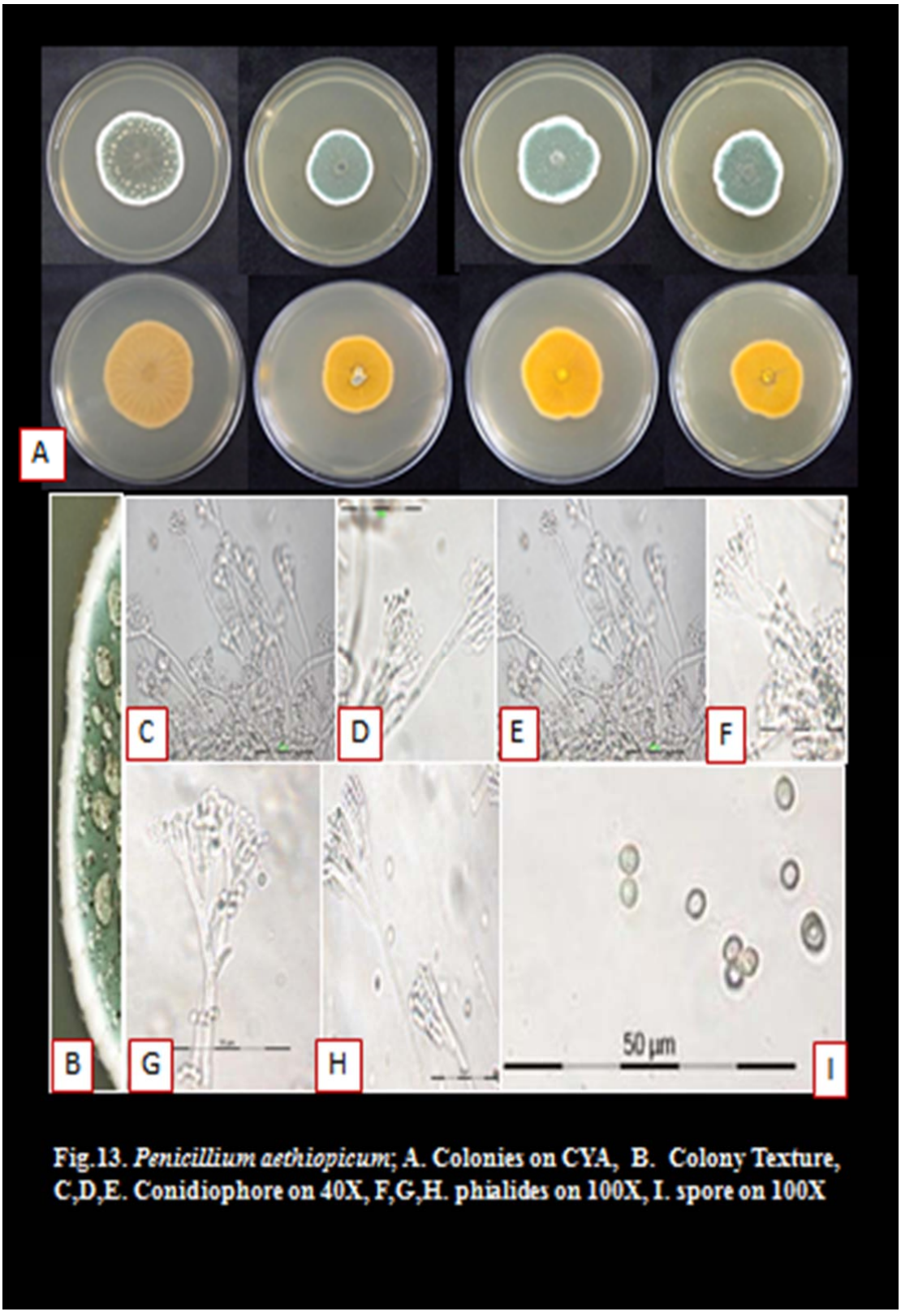

Fig.13. *Penicillium aethiopicum*; A. Colonies on CYA, B. Colony Texture, C,D,E. Conidiophore on 40X, F,G,H. phialides on 100X, I. spore on 100X

4.5.2.***Penicillium chrysogenum*** **(Fig.14):**

In *Penicillium* subgenus *Penicillium* section Chrysogena series *Chrysogena*

Diagnostic features: Roquefortine C & D, chrysogine, penicillin F & G, globose to subglobose to broadly ellipsoidal smooth-walled conidia, relatively short phialides with short broad collula, high growth rate on YES with a yellow reverse and strong sporulation.

Description:

Conidiophores: Bi-, ter- and quarterverticillate, both appressed and divergent rami born from aerial and subsurface hyphae

Conidia: Smooth-walled, globose to subglobose to broadly ellipsoidal, 2.5-4 µm x 2.3-3.5 µm

Phialides: Cylindrical, with short broad collula, 7-9 µm x 2.3-2.5 µm

Metulae: Cylindrical, 8-12 µm x 2.5-4 µm

Rami: Cylindrical, 15-20 µm x 3-4 µm

Stipes: 200-300 µm x 3-4 µm

Synnemata or fasciculation: None

Sclerotia: None

Colony texture: Floccose to velutinous

Conidiumcolour on CYA: Blue green to green

Exudate droplets on CYA: Often present, copious, yellow

Reverse colour on CYA: Cream, yellow, rarely brown

Diffusible colour: Yellow pigment often produced

Odour and volatile metabolites: Hexadecene, Octadecene, Eicosene, Hexadecanoic acid, Docosanol, Pentadecyl-heptafluorobutyrate, Cyclohexadecane, Octadecanoic acid and Griseofulvin.

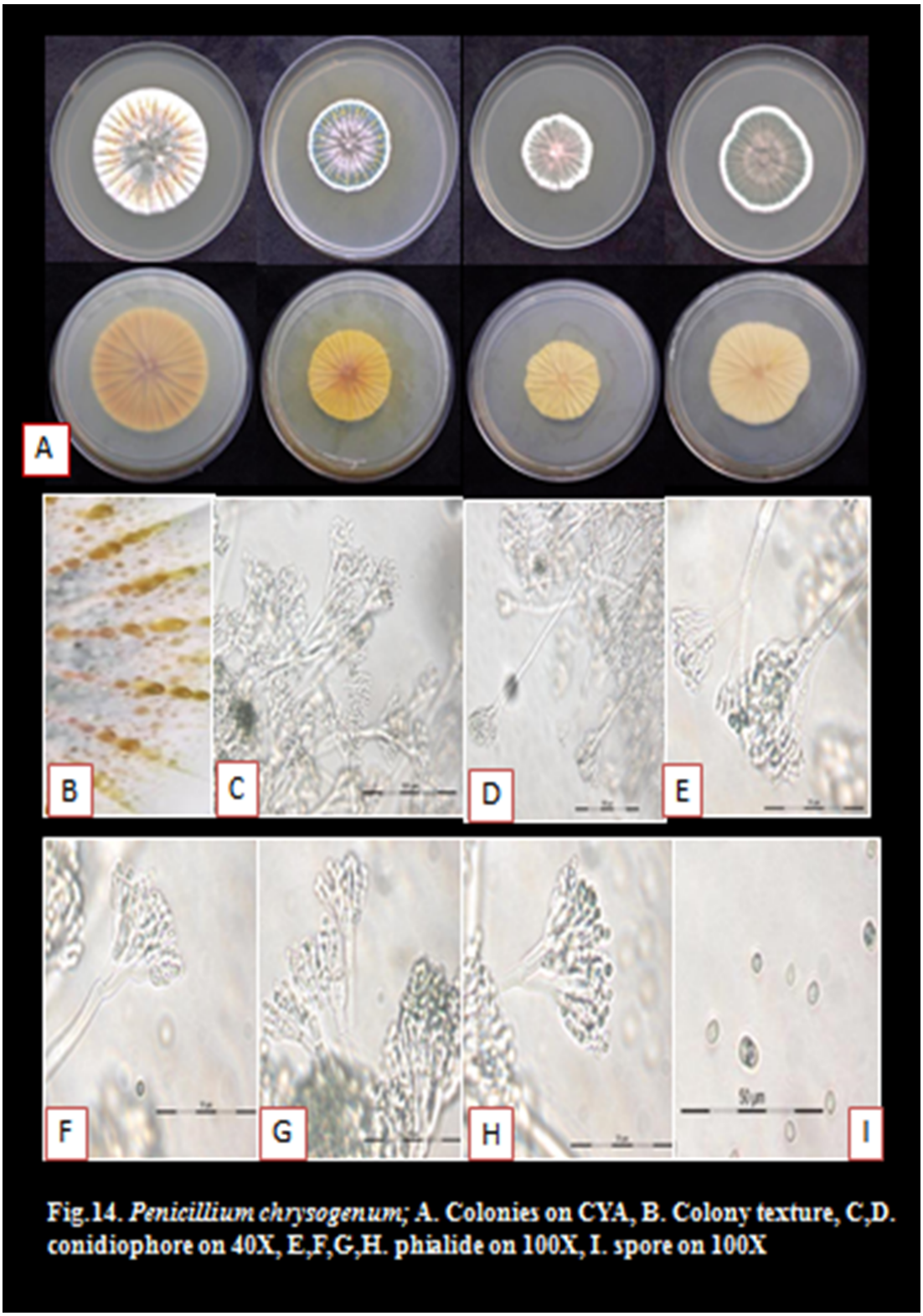

Fig.14. *Penicillium chrysogenum;* A. Colonies on CYA, B. Colony texture, C,D. conidiophore on 40X, E,F,G,H. phialide on 100X, I. spore on 100X

4.5.3. *Penicillium crustosum* (Fig.15):

In *Penicillium* subgenus *Penicillium* section *Viridicata* series *Camemberti*

Diagnostic features: Penitrem A, roquefortine C, terrestric acid, viridicatin, smooth-walled conidia, crustose on CYA and MEA after 7-10 days, high growth rate, good growth on CREA. Strong sporulation and high growth rate on YES agar.

Description:

Conidiophores: Terverticillate, appressed elements, born from subsurface hyphae
Conidia: Smooth-walled, globose to subglobose, 3-4 µm.
Phialides: Cylindrical tapering to a distinct collulum, 9-12 µm x 2.5-3 µm
Metulae: Cylindrical, 10-15 µm x 3-3.5 µm
Rami: Cylindrical, 15-25 µm x 3.5-4 µm
Stipes: Rough-walled, 200-400 µm x 3.5-4.5 µm
Synnemata or fasciculation: Weakly fasciculate
Sclerotia: None
Colony texture: Velutinous to weakly fasciculate, becoming crustose
Conidiumcolour on CYA: Dull green to grey green or blue green at the colony margin
Exudate droplets on CYA: Copious, clear or brown
Reverse colour on CYA: Cream-coloured to yellow brown
Diffusible colour: Pale brown or none
Odour and volatile metabolites: 9,12-Octadecadienoic acid, Hexadecane, Octadecene, E-15-Heptadecenal, Pyrrolo-pyrazine-1,4-dione, Chloro-2-phenoxyethylene, 3-Methyl-4-nitro-benzenamine, Octadecyl trifluoroacetate, 2,5-Piperazinedione, Docosene, 1,2-Benzenedicarboxylic acid and Hexacosene.

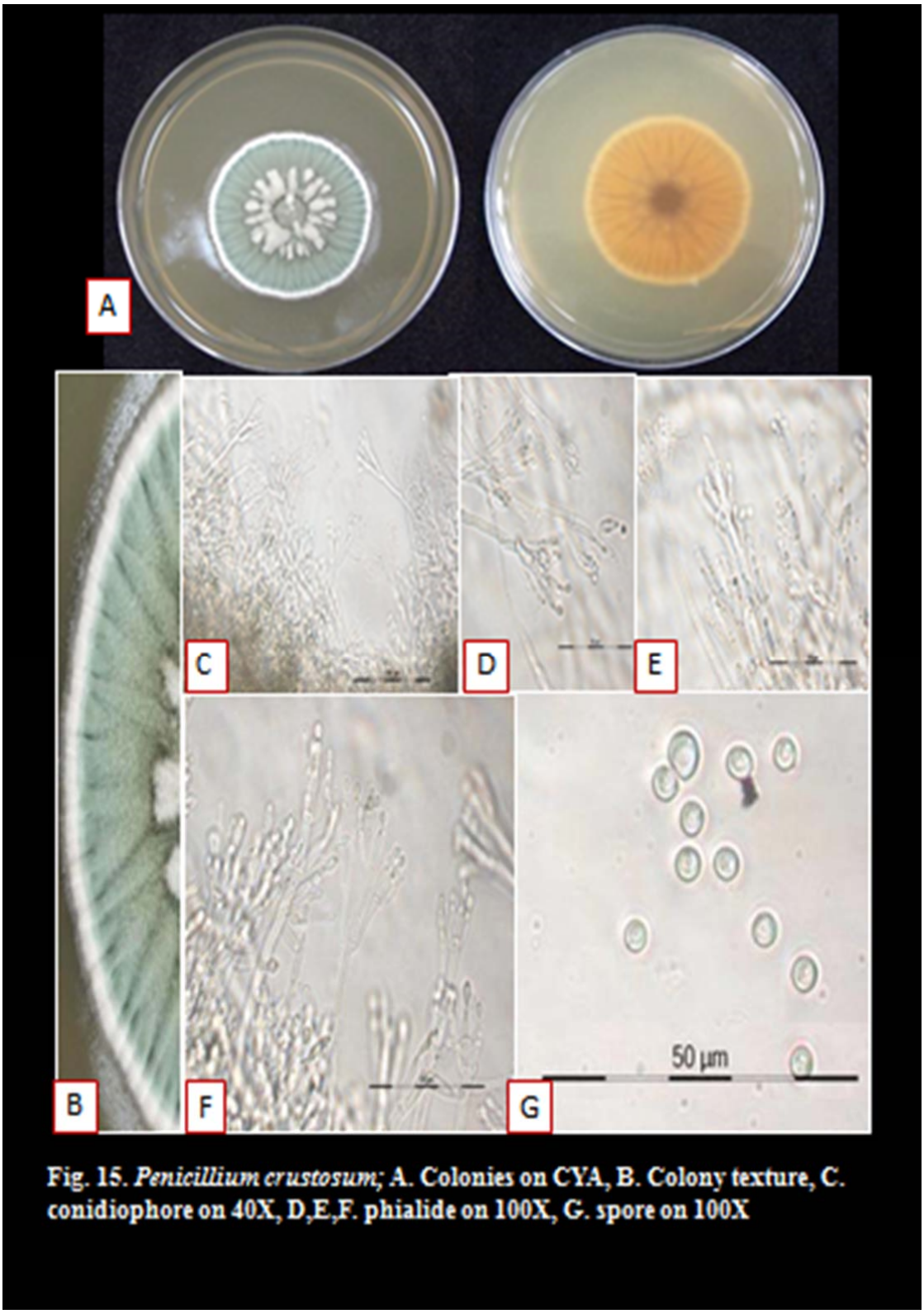

Fig. 15. *Penicillium crustosum;* A. Colonies on CYA, B. Colony texture, C. conidiophore on 40X, D,E,F. phialide on 100X, G. spore on 100X

4.5.4. *Penicillium janthinellum* (Fig.16):

Diagnostic feature: Monoverticilate, ampuliform, long slender collula, pyriform to ellipsoidal conidia.

Similar species: *P. ochrochloron*is closely related *P. janthinellum,* although *P. simplicissimum*in its typical form produces penicilli which are quite different from those of *P. janthinellum,* intermediate between the two species.

Description:

Conidiophore: Monoverticillate from surface or aerial hyphae

Conidia: Spheroidal,2.2-3.0µm in diameter, but sometimes short pyriform to ellipsoidal,2.2-3.0×2.0-2.5µm with smooth to finely roughened walls.

Phialide: ampuliform, 7-11×2.o-2.5µm with long slender collula

Metulae: terminally measuring 12-20×2-2.5µm, sometimes longer if intercalary.

Stripe: smooth and thin walled, easily bent, typically long and slender

Synnemata and fasciation: Absent

Sclerotia: Absent

Colony texture: Floccose usually conspicuous layer of mycillium.

Conidiumcolour on CYA: Greyish green to dull green

Exudate droplet on CYA: Clear to brown or reddish brown.

Reverse colour on CYA: Pale, yellow to yellow brown or reddish brown.

Odour and volatile metabolite: Hexadecene, Octadecane, Pyrrolo-pyrazine-1,4-dione, 2H-Pyrrol-2-one, 1,2-Benzenedicarboxylic acid, Nonadecyl-trifluoroacetate, 2,5-Piperazinedione, 2-benzyl-3,6-dioxo-5-isopropyl-piperazine, Eicosene, 1,2-Benzenedicarboxylic acid, Cyclotetracosane and 17-Pentatriacontene.

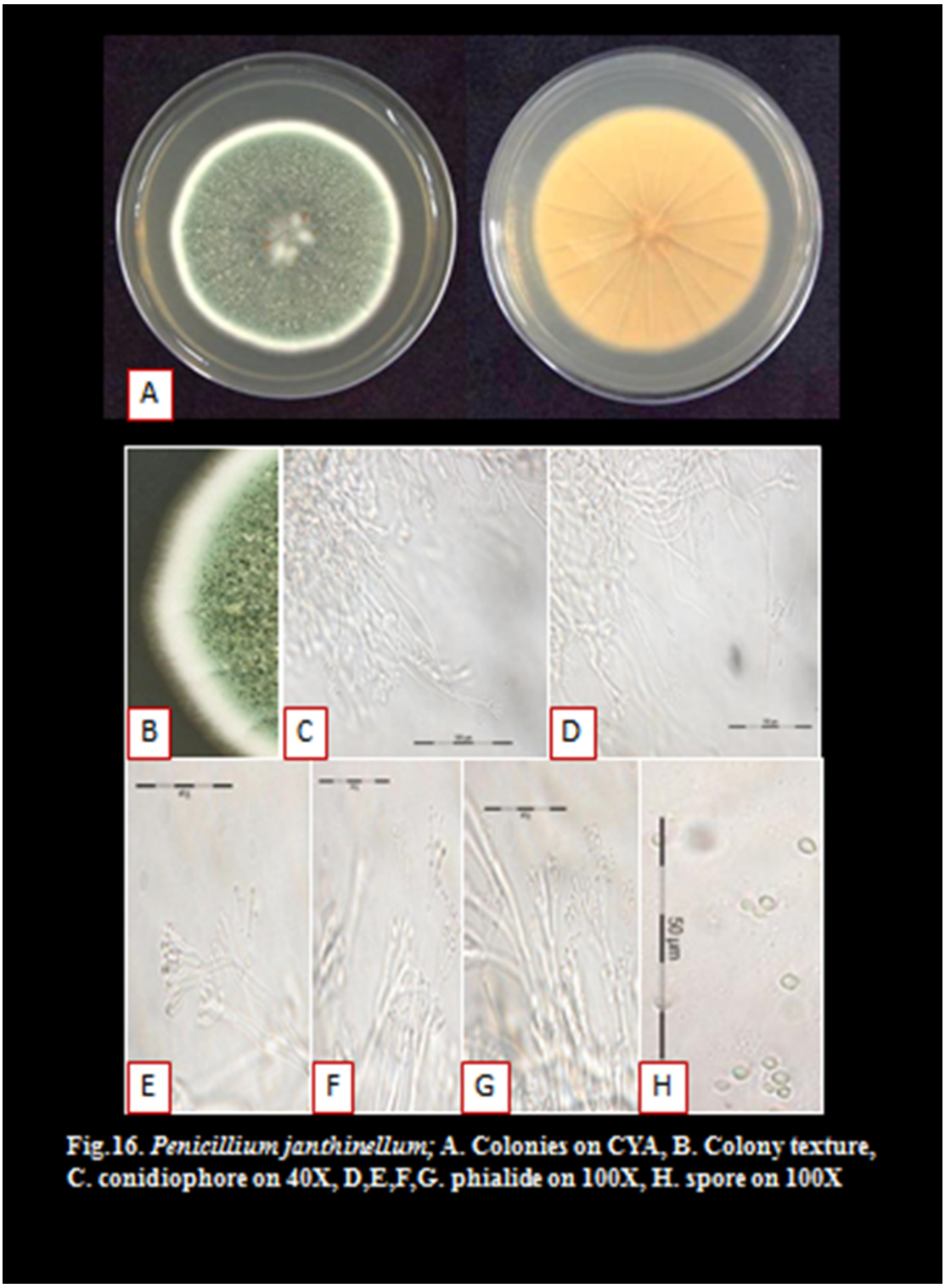

Fig.16. *Penicillium janthinellum*; A. Colonies on CYA, B. Colony texture, C. conidiophore on 40X, D,E,F,G. phialide on 100X, H. spore on 100X

4.5.5. ***Penicillium mononematosum*** **(Fig.17):**

In *Penicillium* subgenus *Penicillium* section *Chrysogena* series *Mononematosa*

Diagnostic features: Fumitremorgins, verrucologen, cyclopaldic acid, divergent structures and often 2 rami, smooth-walled conidia, very good growth at 30°C

Description:

Conidiophores: Terverticillate to quaterverticillate, appressed and divergent elements, born from subsurface hyphae

Conidia: Smooth-walled, subglobose to broadly ellipsoidal, 3.2-3.7 µm x 2.5-3.2 µm

Phialides: Flask shaped with a distinct broad collulum, 7.5- 10 µm x 2.5-3.2 µm

Metulae: Cylindrical, 10-15 µm x 3-4 µm

Rami: Cylindrical, 15-25 µm x 3-4 µm

Stipes: Broad smooth-walled 200-500 µm x 3-4.5 µm

Synnemata or fasciculation: None

Sclerotia: None

Colony texture: Velutinous

Conidiumcolour on CYA: Blue green to green

Exudate droplets on CYA: Copious, clear

Reverse colour: Beige to greyish cream

Diffusible colour: None

Odour and volatile metabolites: 9,17-octadecadienal, Tridecene, Hexadecene, β-Eudesmol, Octadecene, Pyrrolo-pyrazine-1,4-dione, 9,12-Octadecadienoic acid-ethyl ester, Tetradecen-1-ol trifluoroacete, Docosene, 2,5-Piperazinedione, Octacosyl-acetate, Octacosyl-heptafluorobutyrate, Nonadecene, 17-Pentatriacontene and Oleic acid- propyl ester.

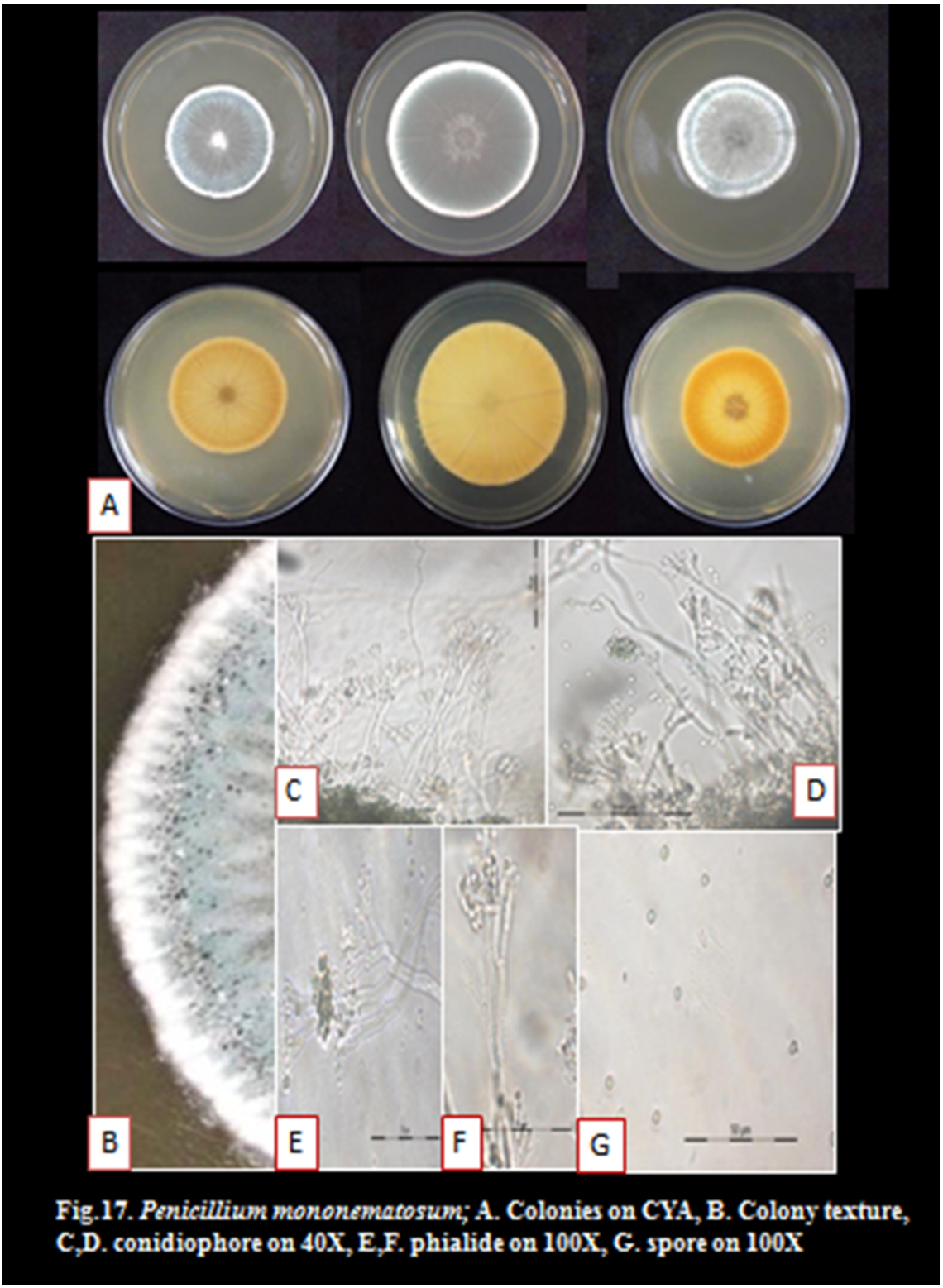

Fig.17. ***Penicillium mononematosum;*** **A. Colonies on CYA, B. Colony texture, C,D. conidiophore on 40X, E,F. phialide on 100X, G. spore on 100X**

4.5.6. *Penicilluium polonicum* (Fig.18):

In Penicillium subgenus Penicillium section Viridicata series Viridicata

Diagnostic features: Penicillic acid, puberuline / verrucofortine, verrucosidin, cyclopenin, cyclopenol, smooth walled conidia,

Description: Conidiophores terverticillate, few biverticillate and quarterverticillate from subsurface hyphae

Conidia: Smooth-walled, globose to subglobose, 3-4 x 2.5- 3.5 µm

Phialides: Flask-shaped tapering to a distinct collulum, 7.5 - 10 µm x 2.5-2.8 µm

Metulae: Cylindrical, 10-13 µm x 2.8-3.5 µm

Rami: Cylindrical, 15-25 µm x 3-3.5 µm

Stipes: 180-400 µm x 3-4 µm, walls smooth to finely roughened

Synnemata or fasciculation: None

Sclerotia: None

Colony texture: Velutinous

Conidiumcolour on CYA: Blue green

Exudate droplets on CYA: Present, clear

Reverse colour on CYA: Pale to cream or yellow brown to red brown

Diffusible colour: None or beige brown to red brown

Odour and volatile metabolites: Hexadecene, Eicosene, Docosene, Pyrrolo-pyrazine-1,4-dione and 9,12-Octadecadienoic acid-ethyl ester.

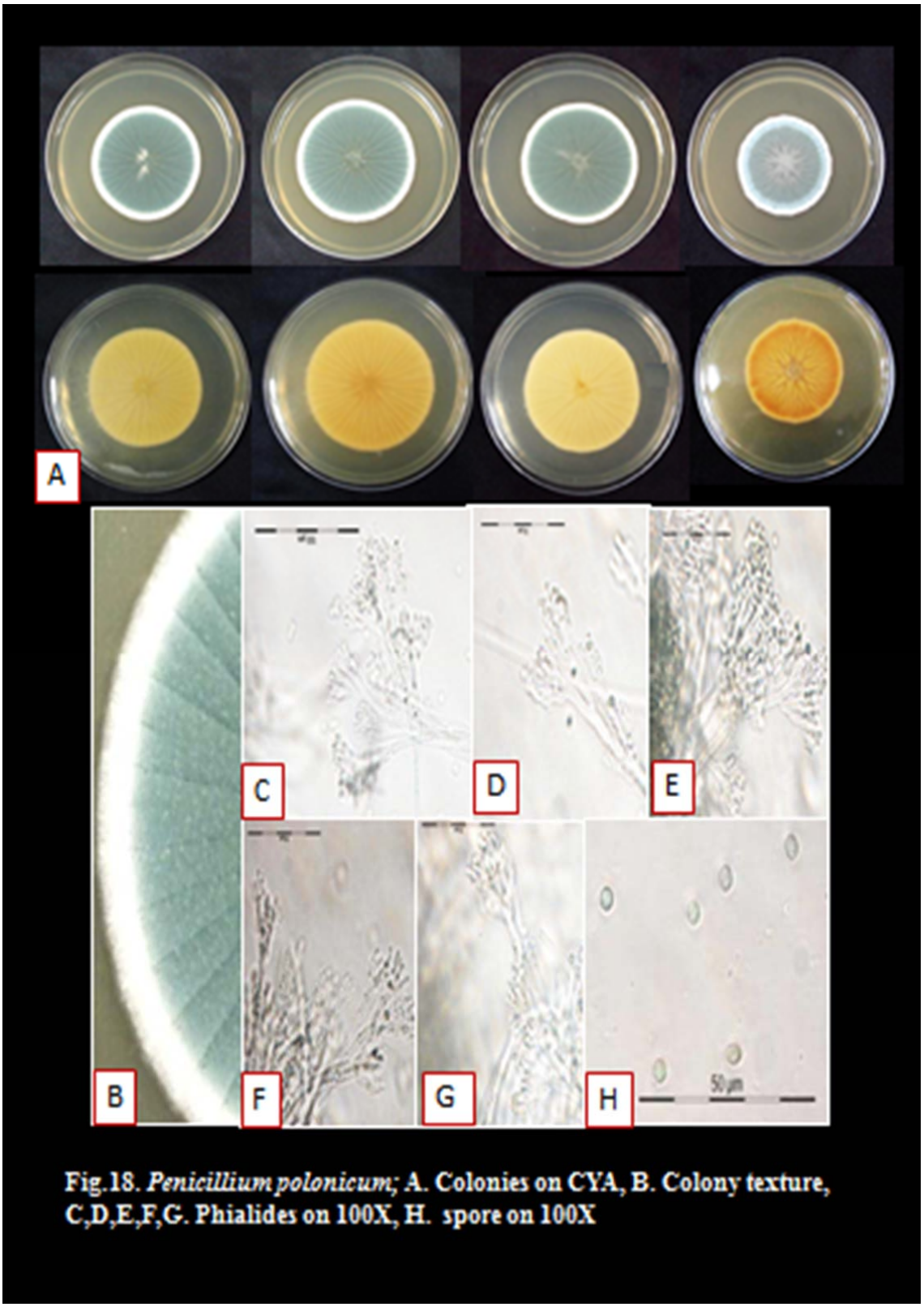

Fig.18. *Penicillium polonicum;* A. Colonies on CYA, B. Colony texture, C,D,E,F,G. Phialides on 100X, H. spore on 100X

4.5.7. *Talaromyces pinopilus* (Fig.19):

Syn. *Penicillium pinophilum*

Dignostic feature: Mitorubrinic acid, biverticillate, stipes smooth walled, conidia smooth, globose to subglobose.

Description:

Conidiophores: Biverticillate;
Conidia: Smooth, globose to subglobose,2–3 × 2–3 µm
Phialide: Acerose
Metulae: three to eight,
Rami: divergent, 10–11 × 2.5–3 µm;
Stripe: Smooth walled
Synnemata or fasciculation: None
Sclerotia: Absent
Colony texture: loosely funiculose and floccose especially in the centre
Conidiumcolour on CYA: Greyish green to dull green
Exudate droplet on CYA: Small clear and red droplets
Reverse colour on CYA: Greyish orange to orange
Odour and volatile metabolite: 9,12-Octadecadienoic acid, 2,5-di-Butyl-Phenol, Hexadecene, Benzofuran-2-one, Octadecene, 1,2-Benzenedicarboxylic acid, Eicosene, Hexadecanoic acid, Docosene, Hexacosene, Methyl 10,12-octadecadinoate and **Griseofulvin.**

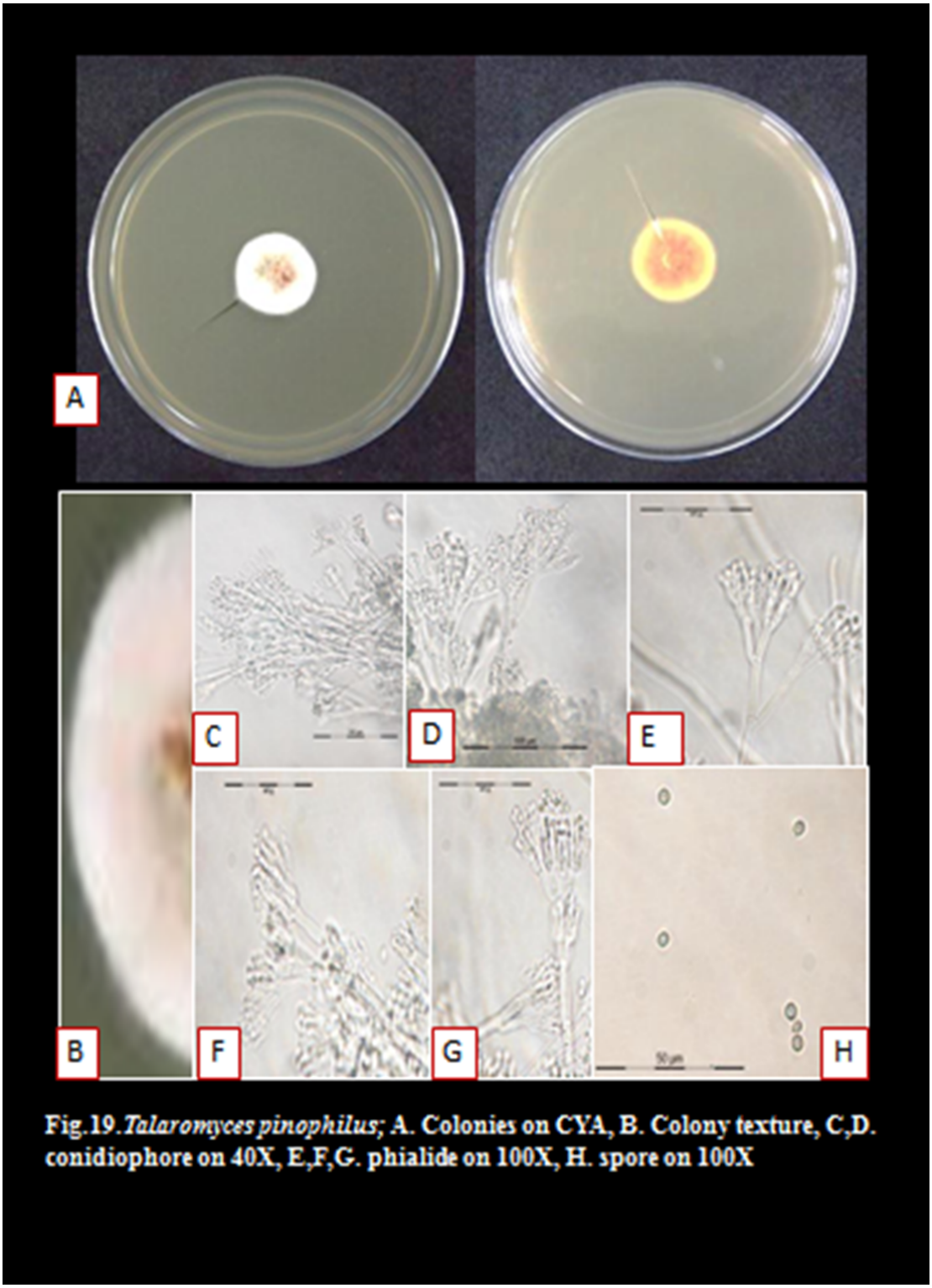

Fig.19.*Talaromyces pinophilus;* A. Colonies on CYA, B. Colony texture, C,D. conidiophore on 40X, E,F,G. phialide on 100X, H. spore on 100X

Penicillium is an anamorph genus and belongs phylogenetically to the family *Trichocomaceae* (Berbee, 1995, Peterson, 2000a). Its main function is the decomposition of organic waste, and cause various pre- and postharvest rots pathogens in agriculturally important crops (Frisvad and Samson, 2004, Pitt and Hocking, 2009, Samson *et al*., 2010), and also produce a range of mycotoxins (Frisvad *et al.*, 2004). Currently the genus contains 354 accepted species (Visagie *et al.*, 2014). Genus *Penicillium* is divided into sections and series due to the large number of species. Though the *Penicillium* species are very common, the genus is well defined, but the species identification is problematic due to less difference in macro- and micro-morphological characters. So cladistic analyses of ribosomal genes and protein coding genes sequences have been used in the molecular studies of several species of *Penicillium*. These studies have resolved some questions of species delineation, but have not provided a robust identification system for the genus *Penicillium*. Therefore, for species delineations, the polyphasic approach was suggested using a combination of morphological, molecular and chemo-profiling data.

The present investigation was carried out with an objective of morphological and molecular characterization of *Penicillium* species. The investigation was started with the collection of soil samples from the ICAR-IARI field. The fungi were isolated from theses samples and the *Penicillium* isolates were separated based on the generic characters and purified through single spore isolation on 2% agar medium. To carry out the morphological characterization of *Penicillium,* Czapek Yeast Autolysate agar (CYA, Pitt, 1979) was recommended. Clemmensen *et al*., (2007) reported that yeast extract-sucrose (YES) medium combined with CYA medium is the best choice of media for identification of *Penicillium* at the species level. Furthermore Pitt *et al*. (1979) reported inoculations are made from spore suspensions in a semi-solid agar solution containing 0.2 % agar and 0.05 % Tween80 for best colony growth. But Visagie *et al.,* (2014) reported CYA as a better medium with 30-37^{o} C incubation to distinguish between *Penicillium* species. The *Penicillium* cultures were grown on CYA in the present study for morphological characterization.

Taxonomically important morphological characters i.e., macromorphology (colony texture, conidium colour on CYA, reverse colour on CYA, exudate colour, margin of colony and pigmentation) and micromorphology (conidiophore structure, shape of phialide, shape and size of spores) were considered for species delineation of genus *Penicillium*. In the present study, nine different species of *Penicillium viz., P. aethiopicum, P. chrysogenum, P. crustosum, P.janthinellum, P. mononematosum, P. oxalicum, P. polonicum, P. singorense* and *Talaromyces pinophilus* (Syn. *Penicillium pinophilum*) were clearly defined using morphological characters. Conidiophore branching patterns were traditionally used in the classification of *Penicillium* (Thom, 1930, Raper and Thom, 1949, Pitt, 1979) with other important microscopic characters for species delineation include the wall texture/ornamentation of stipes and conidia and colour of the conidiophores (Frisvad and Samson, 2004, Visagie *et al.,* 2014, Yilmaz *et al*.,2014). The morphological data obtained in this study was confirmed with earlier studies (Thom, 1930, Raper and Thom, 1949, Pitt, 1979, Frisvad and Samson, 2004, Visagie *et al*., 2014, Yilmaz *et al*.,2014).

In the past, taxonomy of *Penicillium* was based on morphology, but some aspects may vary up to conformed result (visagie *et al*., 2014). To complement the morphological identification, molecular analysis using β-tubulin gene which is an authentic species marker to determine the species characters of *Penicillium.* Even though ITS region is an universal barcode for species identification (Visagie *et al*., 2014a), but for *Penicillium* and many other genera of ascomycetes, the ITS region sequence are not variable enough for distinguishing all closely related species (Skouboe *et al*., 1999, Seifert *et al*., 2007, Schoch *et al*., 2012). Therefore, for Infra-species variation which occurred in β-tubulin gene in some *Penicillium* species were observed in phylogenies of *Penicillium* species (Frisvad and Samson, 2004, Barreto *et al.,* 2011, Peterson *et al*., 2011, Houbraken *et al.*, 2011b,c, Rivera and Seifert, 2011, Rivera *et al*., 2012, Houbraken *et al.,* 2012a, Visagie *et al*., 2013, 2014a, 2014b). This variation must be considered for identification purposes and especially when considering a strain for a new species. In the present investigation, 100% morphological identification matched with the β-tubulin gene identification when blasted both at NCBI and CBS-KNAW database.

In the present investigation, based on the morphological and molecular identification total, nine *Penicillium* species were identified. *P. aethiopicum, P.*

chrysogenum, P. mononematosum, P. polonicum and *Talaromyces pinophilus* had many number of isolates whereas *P. crustosum, P. janthinellum, P. oxalicum* and *P. singorense* had only one isolate each.

Firstly chemical profiling of fungi were based paper chromatography and thin layer chromatography (Frisvad et al., 1989, Lumbsch, 1998) but the methods adapted in mycology were generally for the screening of mycotoxins (Frisvad, 1981, Frisvad and Filtenborg, 1983, 1989, Scott *et al.*, 1970). Although Thin Layer Chromatography (TLC) is no longer considered state of the art for chemical research, it is still a useful technique for detecting coloured or uncoloured extrolites that can be used to confirm the identification of a *Penicillium* strain. For example, *P. brevicompactum* consistently produces large amounts of the colourless mycophenolic acid, and this metabolite will make a green colour reaction with ferric chloride (Clutterbuck and Raistrick, 1933).

For further differentiation and confirmation of the species, all forty one isolates of *Penicillium* were subjected to TLC analysis. In the present study, nineteen *Peniciliium* isolates were selected in cladistics manner from TLC dendogram for further profiling of organic constituents through GC-MS analysis. However, the speciation through TLC banding pattern analysis was not found matching with morphological and molecular identification.

Genus *Penicillium* subgenus *Penicillium* was first time studied for secondary metabolite profiling (Smedsgaard and Frisvad, 1997). It was found that all species produced highly species-specific chemical pattern of secondary metabolites and other secondary metabolites derivatives (Frisvad and Samson 2004, Frisvad *et al*., 2004a, Smedsgaard *et al.*, 2004). Volatile compounds were also used to differentiate many similar species (Larsen and Frisvad, 1995a,b). Chemical profiling of *Talaromyces* (Frisvad *et al*., 1990) and *Penicillium* subgenus *Furcatum* (Frisvad and Filtenborg, 1990, Frisvad *et al*., 2006) disclosed that the *Penicillium* can be segregated based on secondary metabolites, morphology and molecular data.

Volatile secondary metabolites and many common extrolite were been used in *Penicillium* identification (Larsen and Frisvad, 1994). Frisvad (1981) was the first to suggest that extrolites may be used in *Penicillium* taxonomy which was supported by many studies (Frisvad and Filtenborg, 1983, 1989, 1990a).

In the present investigation, from the total nineteen isolates selected for GC-MS analysis, eight isolates *viz.,* S1, S3, S13, S16, S18, S19, S21 and S22 represents *P. aethiopicum*, three isolates *viz.,* 3122, 5080 and S7 represents *P. chrysogenum*, three isolates *viz.,* 5292, 5235 and 6171 represents *P. polonicum*, two isolates *viz.,* 6496 and S15 represents *Talaromyces pinophilus*, 5297 isolate represent *P. janthinellum*, 4255 isolate represent *P. crustosum* and 3212 isolate represent *P. mononematosum.* Different isolates of the each *Penicillium* species show variable peaks and these *Penicillium* species isolates produced the organic constituents *viz.,* antibiotics, hydrocarbons and acids etc. Generally griseofulvin is well known antibiotic isolated from *P. griseofulvum*. But in our studies *P. aethiopicum* produced griseofulvin as a major component.

6. SUMMARY AND CONCLUSION

1. The present work was initiated by the collection of 20 soil samples from the experimental field of ICAR-Indian Agricultural Research Institute (IARI), New Delhi.
2. Fifteen isolates of *Penicillium* were made and the genus was confirmed with the reported literature and named as S series.
3. Twenty six more isolates were added which were taken from Indian Type Culture Collection, Division of Plant Pathology, ICAR-IARI, New Delhi along with ITCC Nos. The total 41 isolates were purified by single spore isolations on 2% agar medium.
4. All these above isolates were characterized using the macroscopic (colony colour and texture; exudate production; soluble pigmentation; reverse colouration and mycelial growth) and microscopic characters (type of penicillus; shape of phialides; conidial shape, size and pigmentation) and made them in to nine different groups.
5. The above groups were identified into nine different species based on reported literature.
6. For confirmation of the above identification, molecular characterization of all the 41 isolates was done using partial β-tubulin gene.
7. The morphological identification was corresponded with the β-tubulin gene sequence based identification. These nine different species were *P. aethiopicum, P. chrysogenum, P. crustosum, P. janthinellum, P. mononematosum, P. oxalicum, P. polonicum, P. singorense* and *Talaromyces pinophilus*.
8. Among them, *P. aethiopicum, P. chrysogenum, P. mononematosum, P. polonicum* and *Talaromyces pinophilus* had many number of isolates whereas *P. crustosum, P. janthinellum, P. oxalicum* and *P. singorense* had only one isolate each.
9. All these 41 isolates were subjected for the production of secondary metabolites through Thin Layer Chromatography (TLC) and Gas chromatography mass spectrometry (GC-MS).

10. An attempt was made to differentiate all the 41 isolates into the species based on their banding pattern but unfortunately the banding pattern was not coincided with the corresponding species.
11. Gas chromatography mass spectrometry (GC-MS) was carried out for 19 isolates and each isolate showed variable peaks which represented the production of organic constituents *viz.,* antibiotics, hydrocarbons and organic acids.
12. In our studies, it was observed that the production of griseofulvin was very high (>80% area in GCMS) in *P. aethiopicum* which is a well-known antibiotic.
13. Based on polyphasic approach using morpho-molecular characters and chemo-profiling data, the above 19 isolates were established into seven different species viz. *P. aethiopicum, P. chrysogenum, P. crustosum, P. janthinellum, P. mononematosum, P. polonicum* and *Talaromyces pinophilus*

Conclusion:

For the above research finding, it can be concluded that *P. aethiopicum, P. chrysogenum, P. mononematosum, P. polonicum* and *Talaromyces pinophilus* are common in Indian soils. β-tubulin gene can be considered as a good marker to differentiate the species of *Penicillium* as it was confirmed from our study when β-tubulin gene sequence based identification was 100% matched with the morphological identification. All the species were also being differentiated based on their secondary metabolites production where in production of griseofulvin was very high in *P. aethiopicum.* Therefore, Polyphasic taxonomy using the combination of all the three methods will provide authentic identification of fungal species.

ABSTRACT

Penicillium is an important fungus which is known to produce enzymes, antibiotics organic acids and mycotoxins. The *Penicillium* species mostly cause post harvest diseases and are able to grow on seeds at low humidity and also cause the devastating rots in food crops as pre- and postharvest pathogens. *Penicillium* is an ascomycetous fungus belongs to the family Trichomaceae which currently contains 354 accepted species. *Penicillium* species are very common and the taxonomic structure of the genus is well defined but the species identification is problematic due to overlapping morphological characters. Therefore, it is necessary to find an alternative method for accurate species identification. An attempt was made for authentic identification of different species of *Penicillium* using polyphasic approach of morphology, molecular and chemo-profiling data. Fifteen isolates of *Penicillium* were collected from the soil samples taken from the experimental field of ICAR-Indian Agricultural Research Institute (IARI), New Delhi. Another twenty six isolates were procured from Indian Type Culture Collection, Division of Plant Pathology, ICAR-IARI, New Delhi. All 41 isolates were characterized based on the distinct macroscopic (colony colour and texture; exudate production; soluble pigmentation; reverse colouration and mycelial growth) and microscopic observations (type of penicillus; shape of phialides; conidial shape, size and pigmentation). Molecular characterization was done using partial β-tubulin gene which is an excellent marker for species resolution of genus *Penicillium*. The morphological characterization was matched with the β-tubulin gene based identification and the nine different species *viz., P. aethiopicum, P. chrysogenum, P. crustosum, P. janthinellum, P. mononematosum, P. oxalicum, P. polonicum, P. singorense* and *Talaromyces pinophilus* were confirmed. All these 41 isolates were inspected for the production of secondary metabolites. Thin Layer Chromatography (TLC) was carried out from crude ethyl acetate extracts and on the basis of banding pattern, the isolates were clustered into different groups. Gas chromatography mass spectrometry (GC-MS) of 19 representative isolates selected from the above groups were analyzed. These isolates were segregated into seven groups based on volatiles compounds produced. Based on the combined data of morphology, molecular and chemo-profiling of 19 isolates of *Penicillium,* seven species viz. *P. aethiopicum, P. chrysogenum, P. crustosum, P. janthinellum, P. mononematosum, P. polonicum* and *Talaromyces pinophilus* were established.

सार

पेनिसिलियम एक महत्वपूर्ण कवक हैं जो एंजाइम, प्रतिरक्षी, कार्बनिक अम्ल एवं कवक विष उत्पन्न करता हैं। पेनीसीलियम प्रजातियाँ अधिकांशतया फसलों के कटाई-उपरांत रोग उत्पन्न करती हैं और कम आर्द्रता पर बीजों के ऊपर उगने में सक्षम हैं तथा भोज्य फसलों में भी , कटाई-पूर्व एवं कटाई उपरांत रोगजनकों के रूप में अत्यंत क्षतिकारक विगलन उत्पन्न कर सकती हैं। *पेनीसिलियम* एक एस्कोमायसीट्स कवक हैं जो ट्राईकोमेसी कुल के अंतर्गत आता हैं, जिसमे इस समय ३५४ स्वीकृत प्रजातियाँ हैं। पेनिसीलियम प्रजातियाँ सामान्यतया पायी जाने वाली प्रजातियाँ हैं और इस जीनस के वर्गीकरण संबंधी संरचना सुस्पष्ट हैं किन्तु आकारिकीय गुणों के अतिव्यापन के कारण प्रजातियों की पहचान करना कठिन हैं। इसलिए, यथार्थ प्रजाति-पहचान हेतु एक वैकल्पिक विधि की खोज अतिआवश्यक हैं। आकारकी, आण्विक एवं रासायनिक प्रोफायलिंग सम्बंधी बहु अवस्था विधि का उपयोग कर *पेनीसीलियम* की विभिन्न प्रजातियों की पहचान का प्रयास किया गया हैं। भा॰ कृ॰ अ॰ प॰-भारतीय कृषि अनुसंधान संस्थान (भा॰ कृ॰ अ॰ स॰), नई दिल्ली के प्रायोगिक प्रक्षेत्र से, मृदा नमूनों से *पेनीसीलियम* के प्रंद्रह विलग एकत्रित किये गए। अन्य २६ विलग भा॰ कृ॰ अ॰ प॰-भा॰ कृ॰ अ॰ संस्थान, नई दिल्ली के पादप रोग विज्ञान संभाग के भारतीय प्ररूप संवर्ध संग्रह से प्राप्त किये गए। सभी ४१ विलगों का, सुस्पष्ट स्थूल (कॉलोनी का रंग एवं बुनावट; बही:स्राव उत्पादन, घुलनशील वर्णकता; कॉलोनी के नीचे, उल्टी ओर का रंग एवं कवकजाल वृद्धि) एवं सूक्ष्मदर्शीय निरीक्षणों (*पेनीसीलियम* का प्रकार; फिएलाइड्स की आकृति, बीजाणु के परिणाम एवं रंग) के आधार पर अभिलक्षणन किया गया। पार्शियल बीटा-ट्यूबलिन जीन, जो जीनस *पेनीसीलियम* के प्रजाति-वियोजन हेतु एक उत्कृष्ट चिन्हक हैं, का उपयोग कर आण्विक अभिलक्षणन किया गया। आकारिकीय अभिलक्षणन का बीटा-ट्यूबलिन आधारित पहचान के साथ मिलन किया गया तथा नौ विभिन्न प्रजातियों अर्थात, *पे॰ एथियोपिकम, पे॰ क्रायोसोजीनम, पे॰ क्रस्टोसम, पे॰ जेंन्थीनेलम, पे॰ मोनोनिमेटोसम, पे॰ ऑक्ज़ेलिकम, पे॰ पोलोनिकम, पे॰ सिंगोरेंस* एवं *टेलेरोमायसेस पाईनोफिलस* की पुष्टि की गई। इन सभी ४१ विलगों का द्वितीयक उपापचयजों के उत्पादन हेतु परीक्षण किया गया। अपरिष्कृत इथायल

एसीटेट निष्कर्षों से महीन पर्त वर्णलेखन (टी एल सी) किया गया तथा पट्टिकाओं के ढंग (बैंडिंग पैटर्न) के आधार पर विलगों का विभिन्न समूहों में समुच्चयन किया गया। उपर्युक्त समूहों से वरण किए गए १९ प्रतिनिधि विलगों का गैस क्रोमेटोग्राफी मास स्पेक्ट्रोमेट्री (जी सी एम एस) विश्लेषण किया गया। इन विलगों द्वारा वाष्पशील यौगिकों के उत्पादन के आधार पर इन्हें सात समूहों में विभक्त किया गया। आकारिकी, आण्विक एवं रासायनिक-प्रोफायलिंग के संयुक्त आंकड़ों के आधार पर *पेनीसीलियम* के १९ विलगों से सात प्रजातियों अर्थात *पे॰ एथियोपिकम, पे॰ क्रायोसोजीनम, पे॰ क्रस्टोसम, पे॰ जेंन्थीनेलम, पे॰ मोनोनिमेटोसम, पे॰ पोलोनिकम* एवं *टेलेरोमायसेस पाईनोफिलस* की स्थापना हुई।

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