INTERNATIONAL JOURNAL OF ADVANCES IN PHARMACY, BIOLOGY AND CHEMISTRY

Research Article

Isolation and identification of soil mycoflora in agricultural fields at Tekkali Mandal in Srikakulam District.

P.K.Ratna Kumar, Hemanth.G *, P.Shiny Niharika and Samuel k kolli.

[3]Department of Botany, Andhra University, Visakhapatnam,

Andhra Pradesh, India – 530003.

ABSTRACT

Soil samples were collected from different rice fields at Tekkali region of Srikakulam District during the months of January 2014 to November 2014 in three intervals. The samples collected in two zones viz rhizoplane and rhizosphere, those collected samples were inoculated in Potato Dextrose Agar (PDA) medium it was supplemented by suitable antibiotics such as penicillin and Streptomycin by using soil dilution method and soil plate method. The present investigation was conducted to find out the fungal diversity in rice fields in and around the tekkali. A total of **168** colonies were isolated. About 18 species belonging to 6 genera of fungi were isolated and identified while 20 strains respectively were left unknown. Identification and characterization of the soil mycoflora were made with the help of authentic manuals of soil fungi. Maximum number of fungal colonies belonged to deuteromycotina (143) and few to zygomycotina (5). Among the isolates *Aspergillus flavus, A.fumigatus, A.nidulans, A.niger, A.terreus, Penicillium chrysogenum, P.frequentens* were predominant.The percentile contribution of the mycoflora were graphically and statistically analyzed.

Key Words: Micro Fungi, Culture Media, Isolation, Fungal Diversity, Tekkali.

INTRODUCTION

There is a vast microbial flora inheriting the earth and they are found in all types of soils[15]. These microbes may interact with the plants resulting sometimes in useful effect and other times in harmful consequences. Fungi are an important component of the soil microbiota[1] and are present as mycelia bits, rhizomorphs or as spores. They play significant role in soils and plant nutrition. Fungi are saprophytic i.e., they live on dead and decaying organic matter, thus breaking it down and converting it to forms that are available to higher plants, as they excrete a wide range of degraded enzymes that attack virtually any organic material. Such degradative activities make fungi essential participants in recycling natural waste in our environment. Unfortunately their degradative proficiency also results in the unwanted growth of fungi that destroy useful materials[2]. Fungi grow on diverse habitats in nature and are cosmopolitan in distribute ion requiring several specific elements for growth and reproduction. In laboratory, these are isolated on specific culture medium for cultivation, preservation, microscopical examinations and biochemical and physiological characterization[2]. The species richness of a fungal community and relative abundance of individual species have been considered as measures of functional activities of the group in the particular habitat[12].

Fungi, bacteria and actinomycetes colonize different habitats and substrates and play substantial role in plant health and productivity besides producing diseases. The role of fungi in the soil is an extremely complex one and is fundamental to the soil ecosystem. They are geographically widely distributed and have been observed in a broad range of habitats principally in soils and decaying vegetation.

Rhizosphere

The rhizosphere is a micro ecological zone in direct proximity of plant roots. It is functionally defined as the particulate matter and microorganisms that cling to roots after being gently shaken in water. The theoretical extent of the rhizosphere is dependent on the zone of influence of the plant roots and associated microorganisms. The rhizosphere is a metabolically busier, faster moving, more competitive environment than the surrounding soil.

Rhizoplane

The rhizoplane is the region around the root epidermis and outer cortex where soil particles, bacterial and fungal hyphae adhere. The functional definition is that after the roots have been shaken briskly in water the remaining microorganisms and soil particles left are considered as belonging to the region of rhizoplane. There are more microbes in the rhizoplane than in the rhizosphere. The diversity of the fungal population is determined by counting the number of colony forming units (CFUs). By spreading the extracted soil microorganisms across agar and counting the number of independent clusters of microorganisms the CFUs were determined. Micro-organisms are abundant where the reliability of the root is compromised. Hence rhizoplane microorganisms tend to be found on older ones rather than younger roots. Bacteria and fungi which are endophytes ie., those that live within the cells of the roots are not considered a part of the rhizoplane.

STUDY SITE AND LOCATION:

Tekkali is a town and a mandal in Srikakulam District in the state of Andhra Pradesh in India. It lies on the coast of Bay of Bengal and is located at 18.6167^{o}N 83.2333^{o}E. the temperature ranges from 18-42^{o}C. Deltaic Alluvial soils, Red Sandy soils and Latirate soils are the major soil types existing. It receives total rainfall of 1162mm with 60% of annual rainfall (705mm normal) during South-West Monsoon season from June to September, and North-East Monsoon provides 277 mm (23.8%) between October and December months. Farmers take up double cropping of paddy with monsoon rainfall and a second crop of sunflower or ground-nut with North-East monsoon rainfall and supplemental irrigation in rabi season.

MATERIALS AND METHODS

Nutrient Medium Used:

A wide range of media are used for isolation of different groups of fungi that influence the vegetative growth and colony morphology. The mediums used were Potato Dextrose Agar (extract from 250g of potato boiled and filtered, dextrose 20g, agar 15g and distilled water 1000ml) [2] . The pH of the medium was maintained at 5.5 being optimal for the growth and spo rulation in a majority of fungi.

Collection of Soil Samples:

The soil samples were collected from five different crop fields at five different locations of Tekkali mandal in Srikakulam district. The samples were collected between the months of January 2014 to November 2014 in three intervals. Most of the fungi are microscopic and show vast variation quantitatively and qualitatively in different sites of collection and at different depths. Therefore soils were collected from a depth of 15cm with the help of a sterilized cork borer pushed horizontally into the ground. The soil caught was emptied into sterilized polyethylene bags. Each sample bag was labeled appropriately by indicating the site of collection, time, date and place of collection. The samples were then taken to the laboratory using sterilized cellophane bags[2]. The collected soil samples along with locations showed in (Table:1).

Isolation of fungi from the soil samples:

The soil dilution[24] and soil plate method[26] on media such as Potato Dextrose Agar used as isolation techniques.

Soil Dilution Plate Method (Waksman,1922): soil dilutions were made by suspending 1g of soil of each sample in 10ml of sterile distilled water. Dilutions of 10^{-3}, 10^{-4} and 10^{-5} were used to isolate fungi in order to avoid over-crowding of the fungal colonies. 1ml of the suspension of each concentration was added to sterile Petri dishes, in triplicates of each dilution, containing sterile Potato Dextrose Agar medium (Fig: 1). 1% streptomycin solution was added to the medium for preventing bacterial growth, before pouring into Petri plates. The plates were then incubated at 28$\pm$2^{o}C for 4-7 days. Organisms were easily isolated because they formed surface colonies that were well dispersed (Fig: 2), particularly at higher dilutions.

Soil Plate Method (Warcup, 1950): About 0.005g of soil was scattered on the bottom of sterile Petri dish and molten cooled (40-45^{o}C) agar medium PDA was added, which were then rotated gently to disperse the soil particles in the medium. The plates were then incubated at 26$\pm$2^{o}C for 4-5 days.

One isolate of each fungal genus from each soil sample were selected at random for further sub-culturing and experiments. The subcultures were maintained on Potato Dextrose Agar Slants.

Inoculating Techniques:

The working benches in the laboratory were thoroughly swapped with methylated spirit soaked in cotton wool, and also a burning blue flame was allowed to sterilize the surrounding air before the inoculation proper. The conical flasks were corked tightly with cotton wool and the Petri dishes were fully autoclaved[2].

Identification of the Soil Fungi:

Generally identification of the fungal species is based on morphological characteristics of the colony and microscopic examinations[5]. The colony growth which includes length and width of the colony, the presence or absence of aerial mycelium, the color, wrinkles furrows and any other pigment production were the macro morphological characters evaluated. Although molecular methods continue to improve and become more rapidly available, microscopy and culture remain commonly used and essential tools for identification of fungal species[5] .The fungi were identified with the help of standard procedure and relevant literature[10,19] .

Staining Technique for Fungi:

Inoculating needles were flamed over the burning Bunsen burner. Then using the needle, a small portion of the growth on the culture plate was transferred into the drop of lacto phenol in cotton blue on the slide. The specimen was teased carefully using inoculating wire loops to avoid squashing and over-crowding of the mycelium[2]. The specimen is observed under the microscope for microscopic identification (Fig: 4).

Statistical Analysis:

Population density expressed in terms of Colony Forming Unit (CFU) per gram of soil with dilution factors. The percent contribution of each isolate was calculated by

$$\% \text{ Contribution} = \frac{\text{Total No. of CFU of an individual Sps}}{\text{Total No. of CFU of all sps}} \times 100$$

CFU – Colony Forming Unit

RESULTS AND DISCUSSION

Soil microbes act as essential determinants of plant community variety and productivity[27]. The environmental factors such as the soil pH, moisture, temperature, organic carbon and nitrogen play an important role in the distribution of mycoflora[9].These are the main factors affecting the fungal population and diversity. The soil mycoflora in five different rice fields viz., Akkavaram, Ayodhyapuram, Parasurampuram, Naupada and Balaramapuram were observed. Soil dilution plate and soil plate methods were used for the isolation of fungi during the present investigation. During a period of 11 months the total number of fungal colonies isolated on Petri plates containing PDA medium were 168. As stated earlier soil dilution plate[25] and soil plate method[26] were employed for the isolation of fungi during the present investigation. A greater number of species and colonies were isolated on soil plates than on dilution plates and further the total number of species isolated decreased with increased dilutions of the samples. The purification of the culture (Fig: 3) was done either by single spore isolation or by culturing of the hyphal tips and was transferred to fresh agar slants of PDA medium. Most of the fungal forms which sporulate heavily were abundant on dilution plates.

Funi are the major decomposers of dead organic matter and contribute significantly in recycling of nutrients in natural and modified ecosystems[8].Altogether five soil samples from five different locations were examined for fungal diversity. The study resulted the presence of 18 species of fungi were identified and characterized from PDA plates (Table: 2). The maximum fungal species belonged to Deuteromycotina (143 colonies) and Zygomycotina (5 colonies) and 20 colonies were left unknown on the plates containing PDA medium (Table: 2)

PDA medium is the most commonly used culture media and was stated to be the best media for mycelia growth by several workers worked with it earlier[14, 23, 28]due to its simple formulation and potential to support wide range of fungal growth. Characterization of the isolates up to genus level and to the species level was made based on the macro-morphological and micro-morphological characters by using authentic manuals of soil fungi.

Our findings are in accordance with the results of Noor Zaman et al.(2012) as the microbial analysis of different samples in rainfed areas of Punjab, Pakisthan. They isolated genera like Aspergillus, Alternaria, Curvularia, Fusarium, Penicillium and Rhizopus. Similar genera were isolated during our investigation. These findings were similar to those isolated by Rasheed et al. (2004)[21]. Aspergillus species particularly like A.flavus and A.niger, Penicillium and Rhizopus were isolated only from the soil where as Alternaria alternata, Curvularia lunata and Fusarium species were obtained from both soil and plant parts. Hence it is considered that isolation of soil samples yielded more fungal species than from plants[20].

In our investigation among the obtained fungal isolates the genera Aspergillus and Penicillium were dominant on media used (Tables: 2). The most common isolates among them viz., *A.clavatus,*

A.flavus, A.fumigatus, A.granulosus, A.nidulans, A.niger, A.restrictus, A.terreus, Curvularia clavata, C.lunata, Fusarium oxysporium, F.solani, Penicillium Chrysogenum, P.frequentens, P.funiculosum, Rhizopus stolonifer, Trichoderma harzianum, T.viride were isolated and characterized. The percent contribution of different soil mycoflora of all the five Rice fields were evaluated. The fungi were mostly observed in the months of June to September as it has been reported that the diversity of fungal population occurred during the monsoon season where the soil moisture was significantly high [3, 4].

Isolation of fungal species from soil samples by repeated screening and plating on starch agar medium by Ratnasri et al. yielded Aspergillus fumigatus and A.niger along with other fungal species[22]. Fusarium solani was isolated from the soils of infected fields and showed 100% frequency[11]. Similarly F.solani was also reported to be cosmopolitan in distribution[17]. Recent study on Soil Microflora in National Parks in Gujarat yielded fungal species like Aspergillus niger and Fusarium species[18].

The conservation of diversity of mycoflora in agricultural fields becomes very essential for the development of sustainable agriculture[9]. Natural and anthropogenic disturbances can alter the species composition or may have negative effect on species diversity of the decomposer fungi[6]. These changes may directly or indirectly affect the vital functions of the soil such as decomposition and mineralization and may result in disturbances. Graphical representation of percent contribution of fungal species diversity in various paddy fields on the media PDA is represented in (Graph:1).

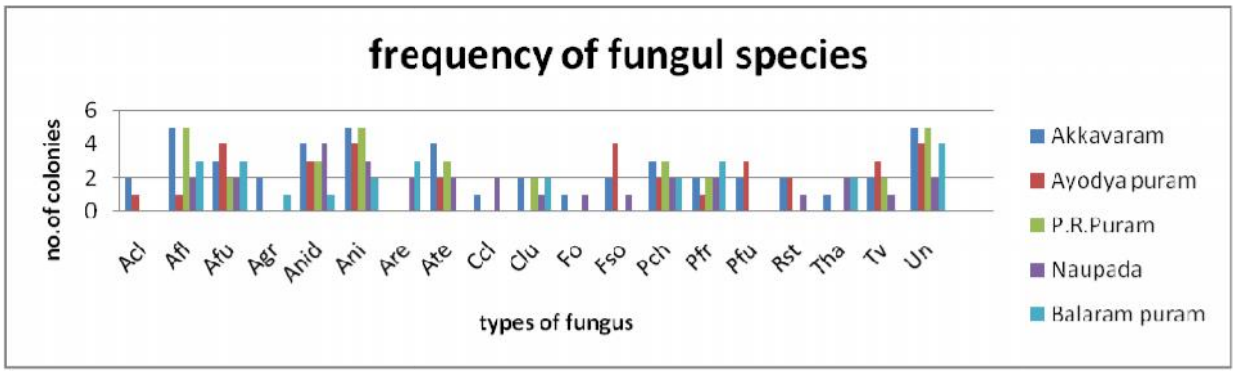
The studies on fungal diversity and percentile contribution and periodic occurrence of soil mycoflora are useful for farmers, agronomists, researchers and microbiologists for future activities in the view of conservation of soil ecosystem, conservation of soil microbial diversity and sustainable agriculture[9].

Table 1
Agricultural soil samples collected from different places in Tekkali mandal.

Sample Number	Agricultural Field	Place
1	Paddy	Akkavaram
2	Paddy	Ayodhyapuram
3	Paddy	Parasarampuram
4	Paddy	Naupada
5	Paddy	Balaramapuram

Table 2
Frequency of mycoflora in different Agricultural Fields as on Potato Dextrose Agar Medium

			Average number of individual colonies																		
			Aspergillus								Curvularia		Fusarium		Pencillium			Rhizopus	Trichoderma		Unknown
	Rice Fields	Total No.of Colonies	Acl	Afl	Afu	Agr	Anid	Ani	Are	Ate	Ccl	Clu	Fo	Fs	Pch	Pfr	Pfu	Rst	Th	Tv	
1	Akka Varam	48	2	5	3	2	4	5	-	4	1	2	1	2	3	2	2	2	1	2	5
2	Ayodya Puram	34	1	1	4	-	3	4	-	2	-	-	-	4	2	1	3	2	-	3	4
3	P.R.Puram	32	-	5	2	-	3	5	-	3	-	2	-	-	3	2	-	-	-	2	5
4	Naupada	30	-	2	2	-	4	3	2	2	2	1	1	1	2	2	-	1	2	1	2
5	Balaram puram	24	-	3	3	1	1	2	3	-	-	-	2	-	-	3	-	-	2	-	4
Total		**168**	3	16	14	3	15	19	5	11	3	5	4	7	10	10	5	5	5	8	20
% Contribution			**1.7**	**9.5**	**8.3**	**1.7**	**8.9**	**11**	**2.9**	**6.5**	**1.7**	**2.9**	**2.3**	**4.1**	**5.9**	**5.9**	**2.9**	**2.9**	**2.9**	**4.7**	**11.9**



Graph 1

Frequency of Fungal species in different crop fields on PDA

A.clavatus, A.flavus, A.fumigatus, A.granulosus, A.nidulans, A.niger, A.restrictus, A.terreus, Curvularia clavata, C.lunata, Fusarium oxysporium, F.solani, Penicillium chrysogenum, P.frequentens, P.funiculosum, Rhizopus stolonifer, Trichoderma harzianum, T.viride

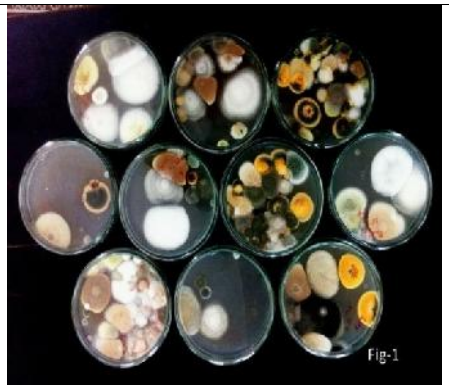
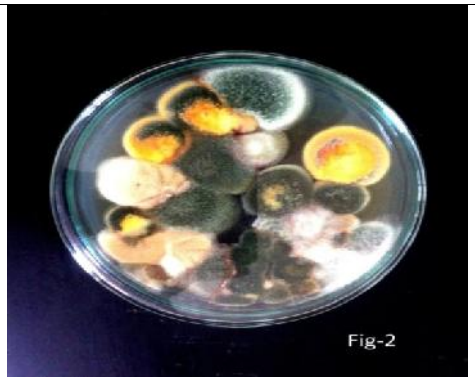
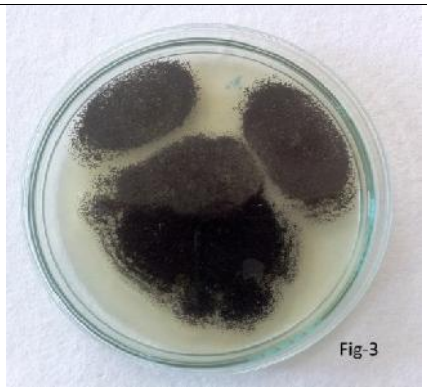
	
Serial dilution of soil 10^3-10^5 in PDA	Selected 10^4 PDA dish
	
Aspergillus niger pure culture	Aspergillus niger

Fig 1 - 4

Isolated Fungi at different stages

CONCLUSION

In the present study the soil sample of five different paddy fields of viz; Akkavaram, Ayodyapuram, Balarampuram, Naupada, Parasurampuram studied for detecting the fungal diversity. The fungal population was observed mostly in the monsoon season as the soil moisture was high. Among the isolates Aspergillus and Penicillium were dominant in all agricultural fields of all areas mentioned due to high sporulation and production of bacterial antibiotics from the Penicillium species and production of different types of toxins from the Aspergillus species may prevent the growth of other fungal species. This study is an effort to understand the soil microbial diversity in the agricultural fields of Tekkali Mandal as soil microflora not only plays an important role in decomposition and contribute to biogeochemical cycling but also are responsible for the prevalence of diseases in the crop fields.

ACKNOWLEDGEMENT

The authors were thankful to Head and faculty members, Department of Botany, Centre of Advanced Studies, Andhra University for their constant encouragement and support.

REFERENCES

1. Ainsworth GC and Bisby GR. Dictionary of the fungi, Commonwealth Mycological Institute Kew, Surrey, 1995; Pp 445.
2. Aina VO, Adewuni AAJ, Hauwa Haruna and Amina Zaraki. Isolation and identification of fungi associated with the deterioration of painted wall surfaces within Kaduna polytechnic. Asian Journal of Medical Sciences, 2011; 3 (6): 250-253.
3. Bissett J and Parkinson D. Functional relationship between soil fungi and environmental in alpine tundra. Can. J .Bot., 1979; 57: 1642-59.
4. Deka HK and Mishra RR. Distribution of soil microflora in jhum fallows in north-east india. Acta Botanica Indica, 1984; 12 (2): 180-184.
5. Diba K, kordacheh P, Mirhendi SM, Rezaie S, Mahmoudi M. Identification of Asergillus species using morphological characteristics. Pal.J.Med.sci., 2007; 23 (6): 867-872.
6. Dong AR, Lv GZ, Wu QY, Song RQ, Song FQ. Diversity of soil fungi in Liangshui Natural Reserve, Xiaoxing'anling forest region, J. Northeast forestry university., 2004; 32(1): 8-10.
7. Eaton AD,Clesceri LS and Greenberg AE.,(Ed.), Standard methods for the examinations of water and waste, 20thEd., American Public Health Association Washington,D.C., 1998.
8. Gadd GM. Mycotransformation of organic and inorganic substrates Mycologist, 2004; 18 (2): 60-70.
9. Gaddeyya G, Shiny Niharika P, Bharathi P and Ratna Kumar PK. Isolation and Identification of soil mycoflora in different crop fields at salur mandal, Adv. Appl. Sci. Res, 2012; 3 (4): 2020-2026.
10. Gilman JC. A Manual of soil fungi, 2nd Indian Edition, Biotech Books, Delhi, 2001.
11. Javed MS, Harif M, Niaz M, Ali I. Impact ofstorage period and temperature on the pathogenic behavior of Fusarium solani on cotton (Gossypium hirsutum L.) seeds. 2008. Mycopath. 6 (1-2): 7-11.
12. Kjoller A, Struwe S. Microfungi in Ecosystems.,fungal occurrence and activity in litter and soil,Oikos., 1982; 39 (3): 389-422.
13. Kjoller A, Struwe S. Functional Groups of Microfungi on decomposing ash litter., Pedobiologia., 1987; 30 (1): 151-159.
14. Maheshwari SK, Singh DV, Sahu AK. Effect of several nutrient media, pH and carbon sources on growth and sporulation of Alternaria alternata, J. Mycopathol.Res., 1999; 37 (1): 21-23.
15. Mukerji KG, Manoharachary C and Singh J. Microbial Activity in the Rhizosphere., Soil Biology., 2006; 7: 1-6.
16. Manickam TS and Venkataraman CR. Effect of continuous application of manures and fertilizers on so physical properties of soil. II under irrigated conditions. Madras Agricultural Journal., 1972; 59: 508-512.
17. Marassas WFO, Burgess WL, Anelich RY, Lamprechi SC, Van Schalkwyk DJ. Survey of Fusarium species Associated with plant debris in South African Soils. S.Ap:J.BoU.54:63-71.
18. Megha Bhutt, sejal patel, Puja Prajapti, Jasral YT. Isolation and Identification of Soil Microflora of National parks of Gujarat, India. Int.J.Curr.Microbiol.App.Sci.2015; 4(3): 421-429.
19. Nagamani A, Kumar IK and Manoharachary C. Hand Book of Soil Fungi, I.K. International Publishing House Pvt Ltd, New Delhi, India. 2006.
20. Noor Zaman, Shakil Ahmed. Survey of Root Rot of Groundnut in Rainfed areas of Punjab, pakisthan. Afr.J.Biotechnol. 2012; 11 (21): 4791-4794.

21. Rasheed S, Dawar S, Ghaffar A. Location of fungi in groundnut seed. Pak.J.Bot.36 (3): 663-668.
22. Ratnasri PV, Lakshmi BKM, Ambika Devi K, Hemalatha KPJ. Isolation, characterization of Aspergillus fumigatus and optimization of cultural conditions for amylase production. International journal of Research in Engineering and Technology.2014; 3 (2): 457-463.
23. Saha A, Mandal P, Dasgupta S, Saha D. Influence of Culture Media and Environmental factors on mycelia growth and sporulation of Lasiodiplodia theobromae (Pat.)., Griffon and Maubl.J.Environ.Biol., 2008; 29 (3): 407-410.
24. Waksman SA. A method for counting the number of fungi in the soil. J. Bact., 1922; 7 (3): 339-341.
25. Waksman SA. Principles of Soil Microbiology. Williams and Wilkins Co., Baltimore, Md., 1927.
26. Warcup JH. The soil plate method for isolation of fungi from soil., Nature., Lond., 1950; 166: 117-118.
27. Wardle DA, Bardgett RD, Klironomos JN, Setala H, Van der Putten WH, Wall DH. Ecological linkages between above ground and below ground biota. Science 2004, 304 (11): 1629-1633.
28. XuSo, Yuan SZ, Chen XC. Studies on pathogenic fungus (Alternaria tennius Nees) of popular leaf blight. J.North East For. Inst., 1984; 12(1): 56-64.