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QUALITATIVE AND QUANTITATIVE ANALYSIS OF MICROFUNGI COLONISING LEAF-LITTER OF *ORYZA SATIVA* L.

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From the leaf- litter of rice, microfungi were isolated by using several complementary cultural techniques. Mycoflora exhibited quantitative as well as qualitative variations. In general, total population and number of species increased with increasing time, however, there was a temporary decrease at second litter stage. Atmospheric conditions and competition among fungi governed the fungal succession and colonisation. The succession occurred not only on the leaf surfaces, but also in the internal colonisers. Qualitatively 4 groups of colonisers were isolated, out of these, first 3 groups, were represented by epiphytic colonisers at early, middle & later stages of decomposition while 4th group was represented by internal colonisers. Deuteromycetes, mainly the dematiaceous hyphomycetes, constituted the major portion of the mycoflora, associated with leaf litter of rice.

Studies on microfungi colonising leaf-litter of various herbaceous plants have so far been followed mostly in temperate areas of the world. (Hudson, 1968; Dickinson and Pugh, 1974). Relatively few such studies on tropical plants are concerned with senescent and/or dead leaves turning to litter (Hudson, 1962; Meredith, 1962; Lal and Yadav, 1964; Khanna, 1970; Sharma and Dwivedi, 1972; Rai, 1973; Kamal and Singh, 1975; Mehrotra and Aneja, 1979; Panwar and Sharma, 1983; Garg and Sharma, 1984, 1985). In most of the studies, relatively little attention has been paid to the quantitative analysis of the mycoflora, possibly because of the disadvantages of the dilution plate technique widely used for quantitative isolations of microfungi. In the present paper, a modified serial wahsings technique has been used in combination with dilution plate on two culture media to obtain a better picture of quantitative spectrum of mycoflora.

MATERIALS AND METHODS

Experimental plots were a part of Botanical Research Farm of the Meerut University, Meerut. The climate is subtropical, semi-arid conditions exist, with extermes of temperature during summer and winter. There are mainly three seasons in the year; rainy (July to September), winter (October to February) and summer (March to June) with marked seasonal variations. Maximum rainfall occurs during July-August, while May and June are the hottest months (max. temp 45°C).

Collection of Dead Leaves - Dead intact leaves of *Oryza sativa* L. were collected at the end of growing season in December, 1988. Leaves were removed from plants with sterile forcep and scissors, placed in sterile nylon-net bags and were left undisturbed in the field under a thin layer of soil. The litter was allowed to decompose under natural conditions. The bags were protected by iron-net huts.

Sampling of Leaf-Litter - Sampling of leaf-litter was done from the bags at different stages of decomposition. First sampling of leaf-litter (L_1) was done just after the collection of dead leaves and subsequent samples were drawn at a regular interval of 20 days until the total fragmentation of litter.

Isolation of Mycoflora - Microfungi were isolated by the following methods-

Dilution Plate (Waksman, 1922) - At each sampling 50 leaf disks (8 mm. diameter) from a number of leaves were cut at random with the help of sterile cork-borer. These disks were shaken in 25 ml aqua-bidest in a 250 ml Erlenmeyer flask for 15 min. The spore suspension was decanted in a separate flask and the same disks were again shaken for 15 min with fresh 25 ml of aqua-bidest. This was repeated six times and finally all leaf-washings were pooled together to make a composite sample which was treated as undiluted suspension. From this 1:10 and 1:100 dilutions were prepared. Ten replicates of 1 ml aliquot from each of the undiluted and diluted suspensions were aseptically made containing 20 ml of cool, molten, potato dextrose agar (PDA) medium having 185 µg/ml streptomycin. The Petriplates were incubated at 25±1°C for 3-10 days. The colonies of microfungi were identified, counted and recorded. The calculations were made to obtain the

population of each fungal species expressed as colonies cm^{-2} leaf-surface area by using following formula-

$$\text{Population of sp 'X' } cm^{-2} \text{ leaf surface area} = \frac{\text{Colonies of sp 'X' in all replicates x Total amount of water x dilution factor}}{\text{Total number of replicates x Total leaf surface area}}$$

Serial Washings (Garg, 1988; Garg and Bhatnagar, 1989) - In this case also, at each sampling 50 more disks were cut at random and placed in a 500 ml sterile Erlenmeyer flask containing 100 ml of aqua-bidest. The flask was shaken at 70 rpm for 30 sec and the suspension was decanted in a separate, sterile, 250 ml flask marked as 'A' leaving the disks in the original flask to which fresh 100 ml of aqua-bidest was added and the flask was again shaken at the same speed for 1 min. In this manner, different serial suspensions were prepared by shaking the disks for 2, 5, 10, 15 and 25 min each time with fresh 100 ml water and the suspensions thus formed were named as suspension B, C, D, E, F and G respectively.

Five replicates from each suspension were prepared with PDA and 5 with leaf-extract dextrose agar medium in the similar manner as for dilution plate. The Petriplates were incubated, colonies were indentified counted and recorded. The population of each fungus was calculated by using the following formula:

$$\text{Population of sp 'X' } cm^{-2} \text{ leaf surface area} = \frac{\text{Total number of colonies of sp 'X' in all SW x Number of SW x Amount of water}}{\text{Total number of replicates x Total leaf surface area}}$$

SW = Serial Washings.

Serially Washed Disks (Harley and Waid, 1955)- 25 leaf disks were taken after serial washings and were allowed to dry on sterile Whatman's filter paper. 5 leaf disks were inoculated on each of the five agar plates and incubated at 25±1°C for 3-10 days. Colonies developed were identified, counted and recorded. Per cent occurrence of each fungus was calculated.

Surface Sterilization (Hering, 1967)- Rest 25 leaf-disks from SW were surface sterilized with $HgCl_2$ solution (0.01% w/v with 5% industrial alcohol) for 1 min. These were successively washed with aqua-bidest 4 times and blotted dry. Five disks were inoculated on each of the five agar plates and incubation was made in the similar manner as for serially washed disks.

RESULTS AND DISCUSSION

Various species of leaf litter fungi observed and/or isolated by various techniques at different stages of leaf-litter decomposition are shown in the Table 1. A perusal of the results obtained during the study, revealed that species composition of the mycoflora varied on different litter stages, exhibiting a fungal succession. There were observed quantitative as well as qualitative differences in leaf-litter flora at different stages of decomposition.

Quantitative Variations in Fungal Population

Variations in species composition and population of fungi at different litter stages isolated by different techniques are presented in Table 3. Initially there was a decrease in total number of species (Table-2) and total population (Table-3) of fungi in L_2 stage and thereafter, a continuous increase was observed. The decrease at L_2 stage was probably due to unfavourable atmospheric conditions. Competition among the species may be another factor. The importance of competition in disappearence of fungi on decaying leaves has also been recognized by Webster (1956, 1957); Hudson and Webster (1958); Meredith (1962); Harper and Webster (1964); Macauley and Thrower (1966); Khanna (1970); Rai (1973); Sharma (1973); Panwar (1979); & Garg and Sharma (1983, 1984, 1985). But later on nature of the substrate may be changed and there occurred succession of fungi with continuous increase in total number of species and population.

All methods, however, yielded different number of fungal species at different litter stages, but all methods showed a similar pattern of fungal colonisation. From all litter stages, maximum number of species were isolated by modified serial washings technique.

During decomposition, some microfungi were dominant and these microfungi greatly affected the total population in either of the direction. These

Table 1: List of leaf-litter fungi isolated by different methods from various litter stages.

S. No.	Species	METHODS					LITTER STAGES				
		SW		ERS							
		PDA	LDA	DP	WC	SSC	L_1	L_2	L_3	L_4	L_5
1.	*Achaetomuim globosum* Rai & Tewari	+	+	+						L_4	L_5
2.	Actinomycetes		+		+				L_3	L_4	L_5
3.	*Alternaria alternata* (Fr) Keissler	+	+	+	+	+		All			
4.	*Alternaria chlamydospora* Mouchacca	+	+	+	+					L_4	L_5
5.	*A. humicola* Oudemans	+							L_3	L_4	L_5
6.	*A. longipes* (Ellis &Everh.) Mason	+	+	+						L_4	L_5
7.	*Aspergillus candidus* Link ex Fries	+		+	+	+				L_4	L_5
8.	*A. oryzae* (Ahlburg in Koaschelt Cohn)	+	+	+			L_1			L_4	L_5
9.	*A. chivelieri* (Mong.)Thom & Church	+	+		+				L_3	L_4	L_5
10.	*A. flavus* Link.	+							L_3		
11.	*A. fumigatus* Fres.	+		+	+	+	All except L_1				
12.	A. *repens* (Corda) De Bary & Woron	+							L_3		
13.	*A. ocharceous* Wilhem		+		+	+			L_3		L_5
14.	*A. luchuensis* Invi	+	+	+	+	+		All			
15.	*A. terreus* Thom	+	+	+	+				L_3		
16.	*A. unguis* (Emil-weil & Gaudin) Thom & Raper	+	+	+					L_3	L_4	L_5
17.	*A. versicolor* Tiraboschi	+		+	+				L_3	L_4	L_5
18.	*Aspergillus* sp. (brown)	+	+						L_3		
19.	*Aspergillus* sp. (perfect state)	+							L_3		
20.	*Aspergillus* sp. (stromatic)	+								L_4	L_5
21.	*Aureobasidium pullulans* (De Bary) Annaud	+	+	+	+		All except		L_2		
22.	*Aureobasidium* sp.		+						L_3		
23.	*Bispora antinnata* (pers. ex. Pers.) Mason.	+	+	+		+	All except		L_2		
24.	*Botrytis cinerea* Pers ex. Fries	+	+	+	+	+		All			
25.	*Candelabrella* sp.	+	+	+	+	+	L_1			L_4	L_5
26.	*Candida albicans* (Robin.) Berkhout	+	+	+	+	+	All except		L_3		
27.	*Chaetomium globosum* Kunze & Schm.	+	+	+					L_3	L_4	L_5
28.	*Chaetophoma* sp.	+	+	+					L_3	L_4	L_5
29.	*Cladosporium cladosporioides* (Fries) de Vries	+	+	+	+	+	All except		L_3		
30.	*C. herbarum* (Pers.) Link ex Gray	+					L_1				
31.	*Coniothyrum* sp.	+	+	+					L_3	L_4	L_5
32.	*Curvularia lunata* (Walker)Boedijn	+	+	+	+	+	All except		L_1		
33.	*C. pallescens* Boedinn	+	+	+	+	+		L_2	L_3		L_5
34.	*C. oryzae* Bugnicourt	+	+	+	+	+				L_4	L_5
35.	Dark sterile mycelium	+	+	+					L_3	L_4	L_5
36.	*Drechslera state* of *Cochliobolus spicifer* Nelson	+	+	+	+	+	All				
37.	D. state of *C. miyabeanus* (Ito & Kuribayashi) Drechster	+	+							L_4	L_5
38.	*Emericella nidulans* (Eidam) ex. Datur Vuill	+	+	+	+				L_3	L_4	L_5
39.	*Fusarium oxysporum* Schl ex. Fries	+	+	+		+	All				
40.	*F. moniliforme* Sheld.	+	+	+	+	+	All				
41.	*F. solani* (Mart.) Sacc.	+	+	+			All				
42.	*Fusarium* sp. (Orange)	+	+	+			L_1			L_4	L_5
43.	*Epicoccum purpurascens* Ehrenb ex Schlecht	+		+	+	+				L_4	L_5
44.	*Gilmaniella humicola* Barron	+		+			L_1			L_4	L_5
45.	*Hyalodendron*. sp.	+	+	+	+	+			L_3	L_4	
46.	*Humicola fusco-atra* Traaen	+		+						L_4	
47.	*Monodictys putredinis* (Wallr.) Hughes.	+	+	+					L_3	L_4	
48.	*Mucor* sp.	+	+	+					L_3	L_4	L_5

Table 1 (Contd.....)

47.	*Monodictys putredinis* (Wallr.) Hughes.	+	+	+					L_3	L_4	
48.	*Mucor* sp.	+	+	+					L_3	L_4	L_5
49.	*M. hiemalis* Wehmer	+							L_3		
50.	*Myrothecium roridum* Tode ex. Fries	+	+	+	+	+				L_4	L_5
51.	*Nigrospora sphaerica* Mason	+	+	+				L_2		L_4	L_5
52.	*Oideodendron griseum* Robak.		+				L_1				L_5
53.	*Penicillium chrysogenum* Thom.	+		+							
54.	*P. luteum* Zukal.	+					L_1				L_5
55.	*P. fellutanum* Biourge.	+		+			L_1				L_5
56.	*P. expansum* Link. ex. Fries.	+	+		+	+		L_2	L_3		L_5
57.	*Periconia paludosa* Muson & Ellis	+								L_4	L_5
58.	*Phoma lingam* (Fr.) Desm.	+							L_3	L_4	L_5
59.	*Pithomyces atro-olivaceus* (Cook & Harkn.) Ellis	+	+	+			All				
60.	*P. chartarum* (Berk. & Cert.) M.B. Ellis	+	+	+						L_4	L_5
61.	*Rhizopus nigricans* Ehrein	+	+		+				L_3		L_5
62.	*Stachybotrys parvispora* Hughes.	+	+		+				L_3	L_4	L_5
63.	Sterile mycelium white			+							L_5
64.	Sterile mycelium with st pycnidia.		+						L_3		
65.	*Stemphylium botryosum* Wallr	+	+						L_3		L_5
66.	*Trichocladium opacum* (Corda) Heeghes	+	+	+			L_1			L_4	L_5
67.	*Trichoderma viride* Pers. ex. Fries	+	+	+					L_3	L_4	L_5
68.	Yest (unidentified)	+	+	+						L_4	L_5
69.	Steel gray col (unidentified)	+	+						L_3		
	TOTAL SPECIES	62	49	46	27	22	21	15	42	49	54

Table 2: Number of fungal species isolated from different litter stages by different methods.

S. No.	Litter stage	SW			DP				WC	SSC
		PDA	LDA	Mean	UND	1:10	1:100	Mean		
1.	L_1	16	13	19	7	6	5	9	5	7
2.	L_2	10	13	13	5	5	6	8	6	7
3.	L_3	33	27	41	11	9	5	13	11	7
4.	L_4	35	29	42	9	12	13	22	14	10
5.	L_5	42	28	47	16	13	11	25	15	10

Table 3: Total population of microfungi in different litter stage.

S. No.	Litter stage	SW		DP			
		PDA	LDA	UND	1:10	1:100	Mean
1.	L_1	516	512	82.8	676	4800	2053.2
2.	L_2	200.8	248.8	20.64	139.2	1920	670.1
3.	L_3	368	325.6	48.96	369.6	1968	795.5
4.	L_4	248.4	105.8	29.92	406.8	7546	2660.9
5.	L_5	383.4	214.8	27.68	420.38	1017.2	3540.0

included *Alternaria alternata, Aspergillus luchuensis, Cladosporium cladosporioides, Drechslera* state of *Cochliobolus spicifer, Fusarium moniliforme, Nigrospora sphaerica, Pithomyces atro-olivaceous.*

In first litter stage, more population was mainly related to the higher number of colonies of *Alternaria alternata, Candelabrella, Cladosporium cladosporioides, Drechslera* state of *Cochliobolus spicifer* and *Fusarium moniliforme*. Some of the dominant species disappeared in second litter stage viz. *Candelabrella*, leading to lesser population. In next litter stages many new species appeared alongwith previous dominant species. *Alternaria humicola* species of *Aspergillus, Chaetomium globosum, Curvularia oryzae, Hyalodendron, Stachybotrys parvispora, Epicoccum purpurascens, Penicillium* spp., *Rhizopus nigricans, Stemphylium botryosum, Trichoderma viride, Chaetophoma, Aureobasidium pullulans* are some of the example of these species (Table 1).

It is also clear from the Table 3 that the results obtained from the serial washings technique seem to be realistic while the populations obtained by dilution plate vary with dilution factor. The populations obtained by different dilutions show great variations,

and therefore, to express their mean does not seem logistic. There are no acceptable standard calculations to express the populations of different dilutions (Dickinson, 1971; Garg, 1980; 1988; Garg and Bhatnagar, 1989). The populations obtained by our modified serial washings technique can be expressed by simple arithmetic means.

Qualitative Analysis of the Mycoflora- At different stages of leaf-litter, the mycoflora exhibited a successional pattern which was observed by different techniques. By the time the leaves reached the soil surface, they were colonized by a variety of parastic and saprophytic fungi. The stage of appearence and duration periods of leaf-litter fungi may be different. The following four patterns of colonisation could be recognised.

Group I- This group included the microfungi which were already present on the dead leaf-surfaces. Some dominant primary colonisers were *Alternaria alternata, Aspergillus luchuensis, Aureobasidium pullulans, Cladosporium cladosporioides, Drechslera* state of *Chchliobolus spicifer, Fusarium oxysporum, F. moniliforme, Gilmaniella humicola, Oideodendron, Pithomyces atro-olivaceous.*

Some microfungi of Group I were considered to be present on dead leaves since the young and green-leaf stages while others come from soil.

The picture of internal colonisers as revealed by surface sterilization technique was more or less similar to that obtained by other methods, but the number of internal colonisers was much less.

In common with other studies, the primary colonisers were from amongst those commonly regarded as "common primary saprophytes" (Hudson, 1968; Norse, 1972; Bell, 1974). In the present investigation, these are *A. alternata; A. pullulans; C. cladosporioides; P. atro-olivaceous.*

All primary colonisers except *Cladosporium herbarum* and *Oideodendron* persisted till last stage of litter decomposition. The long survival of primary colonisers may possibly be due to the competitive advantage which they have by virtue of their ability to colonise at an early stage. Although, there is no evidence at present, their special characteristics of accumulating reserve nutrients in their mycelia might also contribute to their ability to grow and sporulate for a longer period as suggested for primary colonisers of *Dactylis glomerata* by Webster (1956, 1957) and Webster and Dix (1960).

Group II - Members of this group appeared as secondary colonisers on partially decomposed leaves in later litter stages. These include - *Alternaria* spp., *aspergilli, Chaetomium globosum, Hyalodendron, Nigrospora sphaerica, Phoma lingam, Curvularia lunata* and *C. pallescens.*

Besides these new species, some primary colonisers also persisted like *Cladosporium cladosporioides, Drechslera* state of *Cochliobolus spicifer, Fusarium* sp. These species increased with time in their percentage occurrence and some of them persisted till the final litter stage.

Group III - When the litter is going to be digested completely it was colonised by 'Final' colonisers represented mainly by aspergilli, *Alternaria, Curvularia, Candelabrella, Pithomyces, Mucor, Rhizopus, Candida, Chaetophoma, Trichoderma, Stemphylum, Stachybotrys, Humicola.* These are mainly soil fungi some of which were present from initial litter stages.

Group IV - It is a special group of microfungi, which constitute the internal colonisers. The members of this group were isolated by surface sterilization technique. These fungi also belong to the similar genera as the members of the rest of the groups. Some common fungi are *Alternaria alternata, A. luchuensis, Bispora, Candida, Curvularia* sp., *Fusarium* sp., *Epicoccum, Myrothecium.*

When the qualitative analysis of the results obtained from different methods was made, one thing was clear that much higher number of species was isolated by SW technique than DP or any other technique from different litter stages (Table 2). This indicates more efficiency of the former technique.

Number of species decrease in litter stage II with gradual increase till last litter stage. This result is in accord with the populations (Table 4). Maximum number of species were obtained from last litter stage.

Least number of species were isolated by surface sterilization technique and maximum species by serial washings technique when we compared the number of species isolated by SW with that of DP, as a whole it is clear that SW is much more effective as it yielded more number of species (Table 5).

Table 4: Total number of species isolated from different litter stages by all methods

S.No.	Litter stage	Total species
1.	L_1	21
2.	L_2	15
3.	L_3	42
4.	L_4	49
5.	L_5	54

Table 5: Total species isolated from all litter stages by different methods.

S.No.	Litter stage	No. of species		Mean
1.	SW on PDA	62	>	69
2.	SW on LDA	49		
3.	DP	46		
4.	WC	27		
5.	SSC	22		

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