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DIVERSITY OF EPIPHYTIC AND ENDOPHYTIC MICROORGANISMS IN SOME DOMINANT WEEDS

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ABSTRACT

Diversity in epi and endophytic microorganisms from the local weeds is thoroughly studied in this paper. For this purpose, 46 fungal and 19 bacterial strains were isolated from the surfaces and the inner tissues of four dominant agricultural weeds. Leaf wash and homogenized leaf mixture solution were used for the isolations from healthy leaves of four weeds viz. Chenopodium album, Euphorbia helioscopia, Parthenium hysterophorus and Convolvulus arvensis. Our study indicated that complex interactions existed between the host and their epi and endophytic microflora. Each weed has specific bacterial community with the reference of epi and endo phyllosphere. The number and species of bacterial strains varied not only with their host weed plants but also in epi and endo phyllospere. Sørensen's QS of all tested weeds for the endophytic and epiphytic bacterial assemblages was 0.00 that indicated no species overlap / similarity between the communities. Five fungal genera were common as epi and endophytes in all weeds samples: Aspergillus (56% of all isolates), Drechslera (10%), Alternaria (10%) Penicillium (6%) and Cladosporium (4%). Frequency of all five common genera differed significantly among weeds. It was also noted that endophytic fungal communities were not noticeably less speciose than epiphyte communities. Sørensen's QS of E. helipscopia (0.23), C. album (0.37) and C. arvensis (0.46) for the endophytic and epiphytic fungal assemblages was intermediate in the range (0.12–0.79) of previous studies. In case of P. hysterophorus, the value for Sørensen's QS was 0.00 indicating no species similarity. The other identified genera were rare, such as Absidia, Cuvularia, Phoma and Trichoderma.

Key words: Epi and endophytic, fungi, microorganism, phyllosphere, *Aspergillus.*

INTRODUCTION

The phyllosphere of a leaf includes its surface (phylloplane) and the internal tissues colonized by a variety of epiphytic as well as

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endophytic microorganisms respectively, thereby occupying two distinct habitats on the leaf (Andrews, 1996; Carroll *et al.,* 1977; Petrini, 1991). These flora coexist within millimeters of each other, but are usually studied separately and may have important implications for plant health and plant protection (Andrews and Harris, 2000; Arnold *et al.,* 2003; Strobel and Long, 1995; Sturz *et al.*, 2000), microbial biodiversity (Arnold *et al*., 2001; Carroll 1995; Gamboa *et al*., 2002; Hawksworth and Rossman 1997; Petrini *et al.,* 1995) and drug discovery (Strobel and Long, 1995).

Relationships between epiphytes and endophytes have important implications for micro-organisms biodiversity and plant health. It is unclear to what extent plants control which endophytes are able to enter the leaf, and to what extent epiphytes may affect this process (Lebro *et al.,* 2001). The interest shown in the last few years in the study of phyllosphere microbes is due to their interactions with plants, herbivores and pathogens on living leaves which may be involved in the plant immunity system, reabsorption of organic and mineral matters from leachates, redistribution of nutrients prior to leaf fall and participation in the primary degradation of plant tissues (Carroll *et al*., 1977; Cabral, 1985; Lindow and Brandl, 2003; Osono, 2006). Comparison of endophytic and epiphytic flora may help to determine the basis for selectivity. Therefore, the purpose of this study was (1) to search for association of epi and endophytic microorganisms in the weeds habitats and (2) epiphytes and endophytes may potentially be useful for biocontrol study of these weeds. Therefore, with these perspectives it was thought desirable to undertake a preliminary study on the diversity of phyllosphere fungal community of four dominant weed species in agricultural crops of Pakistan.

METERIALS AND METHODS

Sample collection and microbial isolation

The fresh and healthy leaves of four weeds viz. *Chenopodium album, Euphorbia helioscopia, Parthenium hysterophorus* and *Convolvulus arvensis*, were collected from the premises of University of the Punjab, Lahore, Pakistan. Five Leaves of each of 4 weeds were collected from different locations in the university and put separately into sterile bags then taken back to laboratory in less than 2 hours for isolation of epiphytic and endophytic phyllosphere microorganisms.

To analyze epiphytic microflora, leaf washings were used for the isolation. A leaf sample (was shaken for about 1h in 100 ml of sterile distilled water. An aliquot of 1ml from leaf wash was plated on 2% Malt Extract Agar (MEA) medium (g/L): Malt, 20.0; Agar, 20.0 for

fungal isolation and LB Medium (g/L): Peptone, 5.0; Beef Extract, 3.0; Agar, 15.0, was used for bacterial isolation.

For endophytic microflora, leaves of each weed were washed through in running water followed by surface-sterilization in 70% ethyl alcohol (1 min), 2.6% $NaClO_2$ (3 min), and 70% EtOH (1 min). Sterile leaves were ground in blender with 100ml of sterile distilled water to form a homogenized leaf solution mixture. Leaf mixture (1 ml) was then plated on 2% Malt Extract and on LB Medium for fungal bacterial isolation, respectively.

The Petri dishes were incubated for 3-4 days at 25-28^{o}C for the fungal colony count. Bacterial colonies were counted after 24 hours at 37^{o}C and purified for further identification.

Sørensen's quotient of similarity (*QS*) was calculated to examine the similarity of fungal /bacterial assemblages in leaf interiors and on leaf surfaces:

$$QS = 2a / (2a+b+c)$$

Where *a* is the number of common species and *b* and *c* are the numbers of species specific to the interior and the surface, respectively (Osono and Mori*,* 2004). The Sørensen index is a very simple measure of beta diversity, ranging from a value of 0 where there is no species overlap between the communities, to a value of 1 when exactly the same species are found in both communities. The relative abundance/ frequency (%) of each fungal/bacterial species isolated by dilution plating was also calculated as: (Number of colonies of a fungal species/ Total number of fungal colonies) × 100.

Morphological taxonomy of fungal and bacterial isolates

Isolated fungal species were plated onto MEA Petri dishes and incubated for 5 days at 25- 28°C in darkness to observe the colonies' morphology and measure their diameters. A small portion of fungal colony was used to identify the fungal isolates under the microscope on their morphological characters using various mycological keys (Ellis, 1971, Domsch *et al*., 1980, Pitt, 2000). Bacterial strains were identified including pigment, colony form, elevation, margin, texture and opacity (Smibert and Krieg, 1981). In addition, bacterial strains were tested with respect to Gram reaction and biochemical characteristics (Holt *et al.,* 1994).

RESULTS AND DISCUSSION

Diversity of bacteria

Nineteen bacterial species were isolated and identified from phyllosphere of weeds (Table-1). The number and species of bacterial strains varied not only with their host plants but also in epi and endo phyllospere. From *Parthenium hysterophorus* leaves, three ecto (*Peptococcus* sp., *Kurthia gibsonii, Acidovorax facilis*) and two endo

(*Ensifer adhaerens, Acinetobacter calcoaceticus*) bacterial species were isolated. *Chenopodium album* supported *Bacillus farraginis* and *Enterobacter agglomerans* as endophytic bacteria whereas *Curtobacterium albidum* and *Acinetobacter lwoffii* were isolated from epiphyllosphere. *Klebsiella* sp. and *Burkholderia pseudomallei* were only purified from epi phyllospher and *Yersinia ruckeri* and *Corynebacterium minutissimum* from endophyllosphere of *Convolvulus arvensis* (Table-1). In the phyllosphere of *Euphorbia helioscopia,* three bacterial species (*Bacillus farraginis, Kurthia* sp., *Enterobacter agglomerans*) were recorded as epiphytic and three (*Azospirillum lipoferum, Acinetobacter lwoffii*, *Cedecea davisae*) as edophytic bacteria.

Diversity of fungi

We found that all leaves of weed species contained fungal endophytes and epiphytes. A total of 46 fungi were isolated as endo and epiphytic fungal isolates from phyllosphere of four weeds (Table-2). Five fungal genera were common as epi and endophytes from more than one site: *Aspergillus* (56% of all isolates), *Drechslera* (10%), *Alternaria* (10%), *Penicillium* (6%) and *Cladosporium* (4%). The other identified genera were rare, such as *Absidia*, *Cuvularia*, *Phoma* and *Trichoderma.* Frequency of all five common genera differed significantly among weeds (Table-2). For example, *Aspergillus* was a common epiphyte as well endophyte in all test weeds where as *Absidia, Cuvularia*, *Phoma* were only isolated as endophyte in *Chenopodium album* and *Parthenium hysterophoru*s. Sørensen's *QS* of *Euphorbia helioscopia* (0.23)*, Chenopodium album* (0.37) and *Convolvulus arvensis* (0.46) for the endophytic and epiphytic fungal assemblages was intermediate in the range (0.12–0.79) of previous studies. In case of *P. hysterophorus,* the value for Sørensen's *QS* was 0.00 indicating no species similarity.

The aerial parts of living plants including leaves, stems, buds, flowers and fruits provide a habitat for microorganisms termed the phyllosphere. Current study indicated that complex interactions existed among the tested species in epi and endophytic microflora with relation to their hosts. The microscopic examination of the endo and epiphytic phyllosphere gave valuable information on the development, distribution and frequency of the natural mycoflora of weeds leaves surfaces (Table-1 & 2). Total number of microorganisms isolates of phyllosphere differed significantly among tested weeds. Nineteen bacterial species were isolated and identified from phyllospher of weeds (Table-1). The number and species of bacterial strains varied not only with their host plants, but also in epi and endo phyllospere.

Bacteria are considered to be the dominant microbial inhabitants of the phyllosphere, although archaea, filamentous fungi, and yeasts may also be important. These microbes can be found both

as epiphytes on the plant surface and as endophytes within plant tissues (Arnold, *et al.* 2000; Inacio *et al.* 2002; Lindow and Brandl 2003; Stapleton and Simmons 2006). Adams and Kloepper (2002) showed that endophytic bacterial population sizes and structure differed between cotton cultivars, and in cultivars were found to contain endophytic bacteria with one showing a higher colonization level than the others (Elvira-Recuendo and van-Vuurde, 2000). It was concluded from the result that each weed has specific bacterial community with the reference of epi and endo phyllospere. Sørensen's *QS* of all tested weeds for the endophytic and epiphytic bacterial assemblages was 0.00 that indicated no species overlap/ similarity between the communities. Each of the five most common fungal genera [*Aspergillus* (56% of all isolates), *Drechslera* (10%), *Alternaria* (10%), *Penicillium* (6%) and *Cladosporium* (4%)] was more common either outside or inside the leaves, and differences were highly significant in all cases (Table-2). Results also supported that these fungi were predominant in epiphyte and endophytic communities. The presence of these common taxa suggests that either endophytic fungi produce some fast growing spores/or hyphae which come out to the outer leaf surface or epiphytic fungi may have penetrated the host tissues and have colonized internal tissues as endophytes.

Furthermore, unexpectedly, endophytic communities were not noticeably lesser than epiphyte communities. However, in case of *Chenopodium album* and *Convolvulus arvensis*, the number of fungal species found was similar between epiphytes and endophytes (Table-2). However, the possibility of a chance occurrence of certain fungal species on a particular weed cannot be overruled. Sørensen's *QS* of *Euphorbia helioscopia* (0.23)*, Chenopodium album* (0.37) and *Convolvulus arvensis* (0.46) for the endophytic and epiphytic fungal assemblages was intermediate in the range (0.12–0.79) of previous studies on forest tree leaves (Osono and Mori, 2004), while it varies significantly than that of *Pinus resinosa* (0.120) (Legault *et al.* 1989) and *Nothofagus truncata* (0.788) [Ruscoe, 1971].

Abundance of species in both communities approximated a log-normal distribution, as is typical for fungal communities (Dix and Webster, 1995; Gamboa and Bayman, 2001). Frequently recovered fungal species like *Alternaria, Aspergillus, Cladosporium, Penicillium,* and *Trichoderma* spp. from four weeds leaf samples, grow quickly and produce large number of conidia which are easily dispersed and exhibit wide ecological spectrum (Christensen, 1981). Some of these species are able to utilize cellulosic components and gallic acid (Kjøller and Struwe, 1987; Rai *et al.,* 1988) and also found to play important role in primary degradation of plant tissues. *Alternaria alternata, Cladosporium cladosporioides* along with *Fusarium oxysporum* and

Table-1. List of bacterial species isolated from different weeds.

Name of weeds	Epiphytic species	Colony Frequency	Colony %	Endophytic species	Colony Frequency	Colony %	QS
Convolvulus arvensis	*Klebsiella* sp.	4	26	*Yersinia ruckeri*	5	33	0.00
	Burkholderia pseudomallei	3	20	*Corynebacterium minutissimum*	3	20	
Euphorbia helioscopia	*Bacillus farraginis*	8	25	*Azospirillum lipoferum*	4	12	0.00
	Kurthia sp.	7	22	*Acinetobacter lwoffii*	2	6.4	
	Enterobacter agglomerans	7	22	*Cedecea davisae*	3	9.6	
Parthenium hysterophorus	*Peptococcus* sp.	3	18	*Ensifer adhaerens*	3	18	0.00
	Kurthia gibsonii	4	25	*Acinetobacter calcoaceticus*	4	25	
	Acidovorax facilis	2	12				
Chenopodium album	*Bacillus farraginis*	4	25	*Curtobacterium albidum*	6	37	0.00
	Enterobacter agglomerans	3	28	*Acinetobacter lwoffii*	3	18	

Table-2. List of fungal species isolated from different weeds.

Weeds	Epiphtic Fungi	Freq.	Freq. %	Endophtic Fungi	Freq.	Freq. %	QS
Parthenium hysterophorus	*Aspergillus flavus*	6	37	*Aspergillus niger*	2	11	0.00
	Alternaria alternata	3	18	*Aspergillus parasiticus*	6	35	
	Drechslera biseptata	5	31	*Aspergillus reperi*	5	29	
	Mucor sp.	2	37	*Absidia ramosa*	1	5	
				Curvularia clavata	1	5	
				Phoma sp.	2	11	
Convolvulus arvensis	*Aspergillus flavus*	5	23	*Aspergillus flavus*	5	23	0.46
	Aspergillus niger	2	9	*Aspergillus niger*	2	9	
	Aspergillus reperi	4	19	*Aspergillus reperi*	1	7	
	Aspergillus fumigatus	2	9	*Aspergillus fumigatus*	2	15	
	Aspergillus terreus	1	4	*Aspergillus terreus*	2	15	
	Trichoderma sp.	1	4	*Drechslera biseptata*	1	7	
	Drechslera australiensis	1	4	*Drechslera australiensis*	2	15	
Euphorbia helioscopia	*Aspergillus aculeatus*	2	15	*Aspergillus japonicus*	1	9	0.23
	Aspergillus niger	5	38	*Aspergillus aculeatus*	3	27	
	Aspergillus terreus	3	23	*Cladosporium cladosporioides*	1	9	
	Alternaria alternata	1	7	*Aspergillus sydowi*	2	18	
	Drechslera biseptata	2	15	*Alternaria alternata*	1	9	
				Alternaria dianthi	1	9	
				Penicillium spp.	2	18	
Chenopodium album	*Aspergillus phoenicis*	3	18	*Aspergillus phoenicis*	1	10	0.37
	Aspergillus flavus	2	12	*Aspergillus flavus*	4	40	
	Alternaria alternata	9	56	*Alternaria alternata*	1	10	
	Cladosporium sp.	1	6	*Cuvularia clavata*	2	20	
	Penicillium oxalicum	1	6	*Aspergillus reperi*	2	20	

Freq = Frequency

Pestalotiopsis sp. are also recorded as dominant surface and interior colonizers of different tree species leaves (Kayini and Pandey, 2010). In general, these species are extensively reported as common primary saprobes and ubiquitous hyphomycetes from attached leaf surfaces of wide variety of plants throughout the world (Breeze and Dix, 1981; Mishra and Dickinson, 1981; Pandey, 1990; Andrews, 1996; Osono, 2006).

The phyllosphere represents a niche with great agricultural and environmental significance. There is growing evidence for important interactions of phyllosphere microbial inhabitants which may affect the fitness of natural plant populations and the quality and productivity of agricultural crops. Phyllosphere bacteria can promote plant growth and both suppress and stimulate the colonization and infection of tissues by plant pathogens (Lindow and Brandl, 2003; Rasche *et al.*, 2006). Similarly, fungal endophytes of leaves may deter herbivores, protect against pathogens and increase drought tolerance (Arnold *et al.,* 2003; Schweitzer *et al.,* 2006). Epiphytic and endophytic microflora presumably interacts and connects in cross-talk in ways that affect the host plant. Interactions within each community are poorly understood, and interactions between endophytes and epiphytes are completely unexplored. Fungi and bacteria make a complicate linkage of endophytes and epiphytes, and their interactions are also poorly understood. Understanding these phyllospheric communities and their interactions in weeds can improve crops health. Study of phyllosphere microbial communities in weeds, represents one of the most promising and poorly understood areas of agriculture. Understanding the microbial communities and interactions in weeds phyllosphere, can be helpful to improve crop health in sustainable agriculture.

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