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EPIPHYTIC AND ENDOPHYTIC PHYLLOSPHERE MICROFLORA OF *CASSYTHA FILIFORMIS* L. AND ITS HOSTS

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ABSTRACT

In the present study, samples of *Cassytha filiformis* L. and healthy leaves of two of its host plants viz. *Bougainvillea spectabilis* Willd (Nyctaginaceae) and *Citrus aurantifolia* Swingle (Rutaceae) were collected simultaneously from different areas of Lahore, Pakistan. To analyze epiphytic microflora, washings of host leaf/parasite stem were used for the isolation. For endophytic microbes, sterilized homogenized host leaf/parasite stem tissue mixture was plated separately on 2% MEA and LB media for bacterial and fungal isolation. Each fungal colony was purified and identified after 6-8 days on the basis of morphological characteristics. Bacterial strains were identified including pigment, colony form, elevation, margin, texture and opacity. In addition, bacterial strains were tested with respect to gram reaction and biochemical characteristics. The total colonization frequency of the endophytes was maximum for *B. spectabilis* suggesting that this plant tissue harbored more endophytic bacteria than *C. aurantifolia*. On the other hand *Cassytha filiformis* stem, parasitizing *B. spectabilis* and *C. aurantifolia* supported a total of 4 bacterial species as endophytes but different to its host plants. Therefore, Sørensen's quotient of similarity (QS) for the endophytic and epiphytic bacterial assemblages was zero. Overall, the endophyte and epiphyte assemblage of hosts and their parasite showed no overlap.

Key words: Microflora, host, parasite, endophytes, epiphytes, phyllosphere.

INTRODUCTION

The aerial parts of plants including leaves, stems, buds, flowers and fruits provide a habitat for microorganisms termed the phyllosphere. 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, Yadav *et al.* 2004, 2005, Stapleton and Simmons 2006). Bacteria are considered to be the dominant microbial inhabitants of the phyllosphere, although archaea, filamentous fungi, and yeasts may also be important. Phyllosphere bacteria can promote plant growth and both suppress and stimulate the colonisation 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).

A few tropical plants including palms (Southcott and Johnson 1997, Rodrigues 1994), banana (Brown *et al.* 1998) and mangroves (Suryanarayanan *et al.* 1998) have also been

studied for the presence of endophytes in case of attack of parasitic plant. However, Petrini *et al.* (1992) studied host and parasite endophytes altogether. On the other hand, there appears to be no comparative study on endophytes and epiphytes of an angiosperm host and its angiosperm parasite. The study of a host-parasite microflora is expected to throw some light on host specificity of endophytes and epiphytes. Phyllospheric study can also be helpful to find out some relation between parasite plants and their hosts. Hence, we studied *Cassytha filiformis,* an angiosperm parasite and two of its angiosperm hosts belonging to different families for their endophyte and epiphyte microbial assemblages.

MATERIALS AND METHODS

Sample collection: In the present study, stem samples of *C. filiformis* were collected from its two host plants viz. *Bougainvillea spectabilis* Willd (Nyctaginaceae) and *Citrus aurantifolia* Swingle (Rutaceae). Stem portion of the parasite that was not in contact with the host stem were sampled. Healthy leaves of parasitized host plants were also collected. Host leaves and *C. filiformis* stem samples were collected simultaneously from different areas of Lahore, Pakistan. The host and the parasite samples were put separately into sterile bags then taken back to laboratory in less than 2 h for isolation of epiphytic and endophytic phyllospheric microorganisms.

Microbial isolation and identification: To analyze epiphytic microflora of host and parasite, washings of host leaf and parasite stem were used for microbial isolation. Each host leaf and related parasite stem samples were washed separately by shaking for one hour with 100 ml of sterile distilled water. An aliquot of 1 ml from host leaf and parasite stem wash were plated separately on 2% Malt Extract Agar (MEA) medium: Malt 20 g/L, agar 20 g/L for fungal isolation and LB medium: Peptone 5 g/L, Beef Extract 3 g/L, Agar 15 g/L for bacterial isolation.

For endophytic microbial isolations, host leaf and parasite stem were submerged separately in 70% ethanol (v/v) for 1 min to wet the surface, followed by surface disinfection for 3 min in 2% $NaClO_2$. Afterward sample materials were rinsed 2-3 times with sterile distilled water, transferred to sterile filter paper and dried in laminar flow. About 2 g of sterilized sample (host leaf/parasite stem) were ground into paste with 20 mL of sterilized water. For fungal isolation 1 mL of paste solution was added on the MEA medium into a Petri plate and cultured at 25$\pm$ 2°C. For bacterial isolation, 1 mL of paste solution was added on the LB medium and incubated at 37°C. Fungal colonies were counted after 3-4 days. Each fungal colony was purified and identified after 6-8 days on the basis of morphological characteristics (Domsch *et al.* 1980, Ellis 1971 and 1976). 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). Fungi and bacteria were isolated with the surface sterilization method, regarded as inhabitants of the leaf interior of host and parasite, whereas those isolated only with the washing method are regarded as inhabitants of the leaf surface of host and parasite (Osono and Mori 2004, 2005).

Sørensen's quotient of similarity (*QS*) was calculated to examine the similarity of microflora assemblages in host leaf interiors and on leaf surfaces as well as in parasite.

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

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. Frequency of

individual species was calculated as the numbers of colonies of the species grown divided by the total number of all colonies isolated from each sample, expressed as percentage.

RESULTS

Diversity of Bacteria in Host and Parasite Phyllosphere: The leaf of *Citrus aurantifolia* anchoraged two bacterial species as endophytes, while *Bougainvillea spectabilis* had three bacterial strains as endophytes (Table 1). The total colonization frequency of the endophyte was maximum for *B. spectabilis* suggesting that this plant tissue harboured more endophytic bacteria than *C. aurantifolia* (Table 1). On the other hand *C. filiformis* stem, parasitizing *B. spectabilis* and *C. aurantifolia* supported a total of four bacterial species as endophytes but different to its host plants. Therefore Sørensen's *QS* for the endophytic and epiphytic bacterial assemblages was zero (Table 2). Similar observations were recorded in case of epiphytic bacterial species for host plants and parasite. These results strongly suggest the existence of host specificity as well as habitat, among endophytic and epiphytic bacterial species (Tables 1 and 2).

Bougainvillea spectabilis leaves had no fungal species as endophytes (Table 1). Although, some epiphytic fungi were isolated from phyllospere of *B. spectabilis* (Table 2). On the other hand, *C. aurantifolia* showed support in case of both epi and endophytic fungi. The total colonization frequency of fungi was maximum for *C. aurantifolia* suggesting that this plant tissue harboured more fungi (Table 1). *Aspergillus nidulans var. dentatus* was the only fungal species found in epiphytic and endophytic phyllosphere of *C. aurantifolia*. Alternatively, *C. filiformis* stem, parasitizing *B. spectabilis* and *C. aurantifolia* supported a total of five fungal species as epiphytic fungal flora. Sørensen's *QS* for the endophytic and epiphytic assemblages was zero between *C. filiformis* and *B. spectabilis*. Similarity of fungi was also found zero between *C. filiformis* and *C. aurantifolia* (Table 2).

DISCUSSION

The microbial communities of the phyllosphere are diverse, supporting numerous genera of bacteria, filamentous fungi, yeasts, algae and in some situations protozoans and nematodes (Morris and Kinkel 2002, Lindow and Brandl 2003). Bacteria are the most numerous and diverse colonists of leaves, with culturable counts ranging between 10^2 to 10^{12} cells/g leaf (Thompson *et al*. 1993, Inacio *et al.* 2002). In the present study distinct bacterial and fungal species were isolated as endophytes and epiphytes of *C. filiformis* and its two host plants. The plant body of *C. filiformis* is represented by a leafless, yellow green stem that tightly coils around the stem of its host. It also produces haustoria that penetrate the host stem tissue and facilitate the absorption of nutrients from the host. Thus, the parasite is in close contact with its host and consequently, it is exposed virtually to the same type and load of microbes. But in present study host-parasite combination is zero for endophytic assemblages. These results strongly suggest the existence of some degree of host specificity as well as habitat conditions. Plant species, leaf age, leaf position, physical environmental condition, and availability of immigrant inoculum have also been suggested to be involved in determining species of microbes in the phyllosphere (Andrews *et al.* 1980, O'Brien and Lindow 1989, Wilson and Lindow 1994). Leaf surface topography and nutrients present on the leaf surface are generally recognized as important regulators of phyllosphere microbial communities, little research has been done at the whole community level (Hirano and Upper 2000, Yadav *et al*. 2005, Yadav *et al.* 2008).

Table 1. Colonization Frequency of endophytes and epiphytes in host plants.

HOST PLANTS	EPIPHYTIC SPECIES			ENDOPHYTIC SPECIES			
	Bacterial species	**Colony Frequency**	**Colony %**	**Bacterial species**	**Colony Frequency**	**Colony %**	**QS**
Citrus aurantifolia	*Enterobacter agglomerans*	2	9.52	*Micrococcus luteus*	3	14.2	0.0
				Proteus vulgaris	4	14.28	
Bougainvillea spectabilis	*Acetobacter aceti*	4	13.3	*Bordetella pertussis*	3	10	0.0
	Acidovorax temperans	5	16.6	*Ensifer adhaerens*	2	6.06	
				Enterobacter agglomerans	3	10	
Citrus aurantifolia	**Fungal species**	**Colony Frequency**	**Colony %**	**Fungal species**	**Colony Frequency**	**Colony %**	**QS**
	Alternaria alternata	2	11	A*spergillus reperi*	9	50	0.1
	Alternaria sp.	2	11	*Aspergillus flavus*	2	11	
	Aaspergillus fumigatus	2	11	*Alternaria alternata*	1	5	
	Aspergillus flavus	2	11	*Aspergillus nidulans var. dentatus*	6	33	
	Aspergillus nidulans var. dentatus	3	17				
	Aspergillus phoenicis	1	5				
	Curvularia ovoidea	5	29				
Bougainvillea spectabilis	*Aspergillus avenaceus*	1	20		0	0	0.0
	Aspergilllus fumigatus	3	60		0	0	
	Curvularia clavata	1	20				

Table 2. Colonization frequency of endophytes and epiphytes in *Cassytha filiformis* parasitizing different hosts.

PARASITIC PLANT	EPIPHYTIC SPECIES			ENDOPHYTIC SPECIES			
	Bacterial species	**Colony Frequency**	**Colony %**	**Bacterial species**	**Colony Frequency**	**Colony %**	**QS**
Cassytha filiformis parasitizing on *Citrus aurantifolia*	*Pediococcus damnosus*	2	9.52	*Stenotrophomonas maltophilia*	3	14.2	0.0
	Microbacterium lacticum	4	19.04	*Baccillus sp*	2	9.52	
Cassytha filiformis parasitizing on *Bougainvillea spectabilis*	*Lactococcus lactis*	4	13.3	*Spirillospora albida*	2	6.66	0.0
	Pantoea sp	3	10	*Curtobacterium albidum*	4	13.3	
	Fungal species	**Colony Frequency**	**Colony %**	**Fungal species**	**Colony Frequency**	**Colony %**	**QS**
Cassytha filiformis parasitizing on *Citrus aurantifolia*	*Asperigllus niger*	1	25		0	0	0.0
	A*spergillus reperi*	1	25		0	0	
	Fusarium solani	1	25		0	0	
Cassytha filiformis parasitizing on *Bougainvillea spectabilis*	*Aspergillus aculeatus*	10	100		0	0	0.0

Beatie and Lindow (1999) used the term "phyllobacteria" to refer to all the leaf associated bacteria regardless of their location and also illustrated the complexity in the ecology of phyllobacteria when they researched beyond survival strategies to a broader perspective of leaf colonization. They suggested that phyllobacteria employed a number of strategies for colonization that included, modification of the leaf habitat, aggregation, ingression, and egression. In describing these strategies, they found strong evidence in recent literature for a density-dependent interaction among bacterial cells (Swift *et al.* 1994, Beck-VonBodman and Farrand 1995, Greenberg 1997, Pierson *et al.* 1998). Such density-dependent interactions and the ability of bacteria to sense the presence of neighboring cells are often made possible by quorum sensing (QS) which is mediated by the secretion of signal molecules that belong to N-acyl homoserine lactones (HSL) (Swift *et al.* 1994, Greenberg 1997).

Several types of epiphytic bacteria that have associated themselves with the foliar and root surfaces of plants, are dependent on food material shed by the plant as by-products of growth and development. This is evident by results from studies of their metabolic profile (Yadav *et al.* 2008). They may prefer certain types of plants and certain plant parts (Yadav *et al.* 2004, 2005). On foliar surfaces many of these epiphytes are rod-shaped, Gram-negative, pigmented, and fermentative (Thomas and McQuillen 1952, Graham and Hodkiss 1967, Papavassiliou *et al.* 1967, Leben *et al.* 1968). Generally, most epiphytic bacteria do not harm the plant on which they reside, but in some cases, they can either be beneficial or detrimental. Certain strains of *Pseudomonas syringae* and *Erwinia herbicola* also show ice-nucleating traits, and *P. syringae* is also known to be pathogenic. Indeed *P. syringae* seems to remain as resident among epiphytic populations on grasses and trees (Malvick and Moore 1988). *Xanthomonas* isolates also have been found to be active in ice-nucleation (Goto *et al.* 1988). Besides ice-nucleation potential, certain pathogenic *Pseudomonas* species seem to associate epiphytically on their respective host plants. Examination of olive and oleander showed that these plants often harbor the olive knot pathogen *Pseudomonas syringae* pathovar *savastanoi* and other pseudomonads, which represent about 33 percent of the population. Other members of the epiphytic community include *Bacillus* (22 percent) and *Xanthomonas* (10 percent), as well as lesser numbers of *Acinetobacter*, *Erwinia*, *Serratia*, *Lactobacillus*, *Corynebacterium* and *Flavobacterium*, and unidentified nitrogen fixers (Lavermicocca *et al.* 1987). A similar list of bacteria was compiled for olive leaves, with *Pseudomonas syringae* pathovar *savastanoi* and *Erwinia herbicola* being the major epiphytic organisms present (Ercolani 1978). Such close association of the pathogen in the epiphytic state may be important for its long term survival. In the present study, diversity in epiphytic and endophytic bacteria depicted the host and habitat specificity. But in other study by Petrini *et al.* (1992), the endophyte assemblages of fir tree and its mistletoe parasite overlapped by less than 15%.

Colonization ecology of phylloplane and/or phyllosphere fungi principally relates to the prevailing microenvironmental conditions on the leaf surfaces and their physical, chemical and phenological properties which affect the fungal establishment thereon (Pandey 1990, Dix and Webster 1995). The nature and abundance of epiphytic and endophytic leaf fungi have been studied mainly in forests, but their investigation with reference to host parasite are less explored (Heredia 1993, Hata *et al.* 2002). Result showed that microflora exhibited high degree of host specificity. Studies also supported the evidence for host preference within the endophyte and epiphyte

community (Arnold *et al.* 2000). More detailed analyses of the seasonal and leaf age-dependent changes in leaf environmental conditions might provide further insights into the dynamics of endophytic and epiphytic phyllosphere microflora on *C. filiformis* and its host plants.

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