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ANTIFUNGAL AND ANTIBACTERIAL ACTIVITY OF ENDOPHYTIC *PENICILLIUM* SPECIES ISOLATED FROM *SALVADORA* SPECIES

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Abstract

Salvadora persica and *S. S.oleoides* are facultative holophytic plants, well known as miswak, are traditionally used to ensure oral hygiene among Muslim people in Asian and African counties. Species of *Salvadora* have a number of proven pharmacological importance. Besides, terrestrial fungi endophytic fungi are also gaining importance for the isolation of bioactive compounds. In this study 74 samples (root, shoot and leaves) from *S .persica* and *S. oleoides* were examined for endophytic fungi, 22 samples showed presence of *Penicillium* spp., 48 were found positive for aspergilli, whereas 10 samples showed infection of *Fusarium solani,*4 were found infected with *Macrophomina phaseolina* and one with *Rhizoctonia solani.* Most of the *Penicillium* isolated were identified as *P. restrictum, P. citrinum* and *P. canescens.* In dual culture plate assay out of four *Penicillium* isolates tested, *P.citrinum* and one isolate of *P.restrictum* caused growth inhibition of all four test root rotting fungi, *Fusarium solani, F. oxysporum, Macrophomina phaseolina* and *Rhizoctonia solani.* Culture filtrates of *Penicillium* spp., were also evaluated against four common laboratory bacteria namely *Bacillus subtilis, Staphylococcus aureus, Salmonella typhimurium* and *Escherichia coli* and above mentioned root rotting fungi. Culture filtrates of endophytic *Penicillium* spp., also showed significant antibacterial and antifungal activity. Secondary metabolites of endophytic *Penicillium* spp., offer an exciting area of research for the discovery of novel antimicrobial compounds.

Key words: *Penicillium*, Endophytic, *Salvadora* species, Antifungal, Antibacterial.

Introduction

The micro-fungi have been the source of novel and pharmacologically active compounds over decades (Hormazabol *et al.,* 2005; Teakahashi & Lucas, 2008). Among these genus *Penicilium* has been recognized as a rich source of bioactive metabolites (Fill *et al.,* 2007). The genus comprises of more than 200 reported species and most of them are common soil inhabitant, food borne contaminants as well as food ingredients used in the production of cheese or sausages (Domsch *et al.,* 1980; Ali *et al.,* 2011). *Penicilium* spp., produce a range of medicinally important metabolites including antimicrobial (Lucas *et al.,* 2007), antifungal (Nicoletti *et al.,* 2007), immunosuppresents (Kwon *et al.,* 2002), anticancer berkelic acid, polyketides with HIV integrase inhibitory activity and insecticidal (Singh, 2003, Stierle *et al.,* 2006) and nematicidal (Qureshi *et al.,* 2012).

The *Salvadoraceae* is a small family comprising of three genera (*Azima, Dobera* and *Salvadora*) and 12 species which are distributed mainly in the tropical and sub-tropical Asia and Africa (Willis, 1973). *Salvadora* is the only genus of this family found in Pakistan with two species, *Salvadora persica* L*.,* and *S. oleoides* Decne (Qureshi, 1972; Perveen & Qaiser, 1996; Korejo *et al.,* 2010). The natural habitats of *Salvadora* are near mangroves, in saline land, swamps, thorn shrubs, deserts and flooded plains. *Slavadora* spp., are also found near riverbanks where ground water level is high indicating their tolerance to a wide range of water, soil pH and salinity (Zodape & Indusekhar, 1997). *Salvadora* plants most commonly known as miswak are traditionally used to ensure oral hygiene in Muslim people in developing countries (Chelli-Chentouf *et al.,* 2012). The *Salvadora* species have a number of proven pharmacological importance (Almas, 2002; Almas & Al-Zeid, 2004; Darmani *et al*., 2006; Chelli-Chentouf *et al.,* 2012).

Plants are naturally associated with mutualistic microbes that include endophytes. Endophytes are diverse microbes, most commonly fungi and bacteria (Hallmann & Sikora, 1996), which live in plants for at least part of its life without causing visible disease (Clay & Schardl, 2002; Clay, 1991; Kado, 1992). Considerable evidence has now been accumulated in recent years to support and identify the benefits associated with the use of endophytes in crop protection and pharmacological application (Afzal *et al.,* 2013; Sun *et al.,* 2011; Khan & Lee, 2013; Oliveira *et al.,* 2009). Biocontrol and plant growth promoting potential of endophytic *Pseudomonas* and antioxidant activity of some endophytic fungi associated with *Salvadora* have been reported (Dhankhar *et al.,* 2012; Ehteshamul-Haque *et al.,* 2013). However, biological activity of endophytic *Penicillium* species has not been investigated so far. The present report describes the isolation and identification of endophytic fungi associated with *Salvadora* species in Sindh and antimicrobial potential of *Penicillium* spp.

Materials and Methods

Collection of plant samples: Root, shoot and leaves from healthy *Salvadora persica* and *S. oliedes* were collected from Karachi University Campus, Makle, Jampeer, JangShahi from Thatta district, Jamshoro distiric, Sanjar,

Sahra and Moro from district Noushra Feroz for the isolation of endophytic fungi and kept at 4°C until isolation was made within 24 hours.

Isolation and identification of endophytic fungi: One gm of plant sample (root, leaves and stem) was separately washed with running water then sterilized with 1% Ca $(OCl)_2$ for 3 minutes followed by 70% alcohol for 2-3 minutes and finally washed with distilled water for about one minute. They were then chopped in to small pieces in blender with 50 ml of water so as to give the dilution of 1:50. Dilutions of each sample was prepared up to $1:10^4$ and 0.1 ml suspension was transferred onto a Petri dish containing Potato Dextrose Agar supplemented with penicillin (100000 units/liter) and streptomycin (0.2gm/liter). The plates were incubated at 28°C for 5 days and fungi were identified with reference to Barnett & Hunter (1998); Booth (1971); Domsch *et al.,* (1980), Dugan (2006), Ellis (1971); Gilman (1957); Nelson *et al*., (1983); Raper & Fennel (1965) and Raper & Thom (1949).

***In vitro* dual culture plate assay for determining the antifungal activity of *Penicillium* spp.:** Antifungal activity of endophytic *Penicillium* was determined against four common root rotting fungi viz., *Macrophomina phaseolina, Rhizoctonia solani, Fusarium solani* and *F. oxysporum.* A 5 mm agar disc of test *Penicillium* was inoculated on one side of 90 mm Petri dish containing Czapek's Dox Agar pH 7.2. On the other side of same Petri dish, a 5 mm disc of test pathogen was inoculated and incubated at 28°C for 5 days. Zone of inhibition was measured daily, averaged and expressed in mm. There were three replicates of each test and repeated twice.

Preparation of culture filtrates: Among the endophytic fungi isolated from different parts of the plants, *Penicillium* spp., were selected for further study, since *Fusarium, Rhizoctonia* and *Macrophomina* are well known plant pathogens and infect a wide range of plants. Whereas *Aspergillus flavus* is famous for producing mycotoxins and *A. niger* did not show significant antifungal activity in dual culture plate assay. Test *Penicillium* spp., were grown in 500 ml conical flask containing 200 ml Czapek's Dox broth, plugged with cotton wool and autoclaved at 121°C for 20 minutes. After cooling the medium, each flask was inoculated with 5 mm disc, cut from the margin of vigorously growing culture of test fungi. These flasks were incubated for 15 days at room temperature (25-30°C). After 15 days, test fungi were filtered and culture filtrates were collected in sterile flasks. The culture filtrates were then exposed to chloroform vapors to kill any propagules of *Penicillium* or any contaminant, if any. Sterility of culture filtrates was checked by spreading 0.1 ml each on Potato Dextrose agar and nutrient agar with five replicates. Culture filtrates were considered sterile when growth of any fungus or bacterium was not observed after 5 days incubation.

***In vitro* antifungal activity of culture filtrates of *Penicillium* spp.:** To determine the antifungal activity of extracellular metabolites of *Penicillium* spp., thick sterile filter paper discs were impregnated with sterile culture filtrate of each *Penicillium* spp., at 20, 40 and 60 µl/disc and dried. These discs were placed at different position of periphery of plates containing Czapek's Dox Agar. In the centre of Petri Dishes a 5 mm disc of test fungus was inoculated. Discs impregnated with sterile broth of Czapek's Dox broth served as control, whereas carbendazim at 20 µg/disc served as positive control. Petri dishes were incubated at 30°C for 5-7 days and distance between test fungus and disc was considered as zone of inhibition (Qureshi, 2003).

Antibacterial activity of culture filtrates of *Penicillium* spp.: Antibacterial activity of culture filtrates of *Penicillium* spp., was determined against four common laboratory microbes viz., *Bacillus subtilis, Staphylococcus aureus, Eschirechia coli* and *Salmonella typhimorium.* Bacterial lawn of test bacterium was prepared by spreading the bacterial suspension on Trypticase Soy Agar plates with the help of sterilized cotton and disc of the culture filtrates and control were placed at different positions in the Petri dish. Streptomycin (20 µg/disc) served as positive control. Plates were incubated at 30°C for 2-3 days and diameter of zones of inhibition was recorded, averaged and expressed in mm. The experiment was repeated twice with three replicates.

Results

Out of 74 samples (root, stem and leaves) from *S. persica* and *S. oleoides* examined for endophytic fungi, 22 samples showed presence of *Penicillium* spp., 48 were found positive for aspergilli, whereas 10 samples showed infection of *Fusarium solani,* 4 were found infected with *Macrophomina phaseolina* and one with *Rhizoctonia solani. Aspergillus niger* and *A. flavus* were two species of *Aspergillus* isolated, however *A. niger* was isolated in greater number than *A. flavus* (Table 1). *Macrophomina phaseolina* a soilborne fungus was not only isolated from inner roots but also isolated from stem of *S. persica* plants collated from Karachi University campus and Makle. Whereas *F. solani* was found associated with roots of *S. oleoides* collected from Karachi University (Table 1). Isolates of *Penicillium* spp., were identified as *P. restrictum, P. citrinum* and *P.canescens.* However, *P. restrictum* was isolated at higher frequency followed by *P. citrinum* (Table 1). Four isolates of *Penicillium* belonging to three species were tested against four root rotting fungi *M. phaseolina, R. solani, F. solani* and *F. oxysporum in vitro.* In dual culture plate assay *P. citrinum* (EFS-69) and *P. restrictum* (EFS-38) inhibited the radial growth of all the four test fungi by producing zone of inhibition. Whereas *P. canescens* (EFS-55) inhibited the growth of *M. phaseolina* and *F. oxysporum* (Table 2).

Cell free culture filtrates of all the four test isolates of *Penicillium* also showed significant antifungal activity against these root rotting fungi at 60 µl/disc (Table 3). Cell free culture filtrates of these endophytic *Penicillium* spp., also showed significant antibacterial activity against four test bacteria *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Salmonella typhimurium* at 60 µl/disc (Table 4). *Penicillium citrinum* at 40 µl/disc was also effective against these bacteria (Table 4).

Table 1. Endophytic fungi isolated and identified from *Salvadora* species.

S. No.	Host	Plant part	Fungi isolated	Locality
1.	*S. oleoides*	Root	*Fusarium solani*	Karachi University
2.	”	”	*Aspergillus flavus*	”
3.	”	”	*F. solani*	”
4.	”	”	*F. solani, Aspergillus niger*	
5.	”	”	*F. solani*	”
6.	”	”	*F. solani, A.flavus*	”
7.	”	”	*F. solani*	”
8.	”	”	*Macrophomina phaseolina*	”
9.	”	”	*F. solani, Rhizoctonia solani*	”
10.	”	”	*F. solani*	”
11.	”	”	*F. solani*	”
12.	”	”	*F. solani*	”
13.	”	”	*A. niger, A. flavus*	”
14.	”	”	*A. niger*	”
15.	”	”	*A. flavus*	”
16.	”	”	*A. flavus*	”
17.	”	”	*A. niger*	”
18.	”	”	*Penicillium citrinum*	”
19.	”	Leaves	*P. citrinum, A. niger*	”
20.	”	”	*A. niger*	”
21.	”	”	*P. citrinum*	”
22.	”	”	*A. niger*	”
23.	”	”	*P. citrinum*	”
24.	”	”	*P. citrinum*	”
25.	”	Stem	*Penicillium canescens*	”
26.	”	”	*P. canescens*	”
27.	”	”	*A. niger*	”
28.	*S. persica*	”	*A. niger*	”
29.	”	”	*A. flavus*	”
30.	”	Stem	*A. niger*	”
31.	”	”	*A. niger*	”
32.	”	”	*P. restrictum, A. niger*	”
33.	”	”	*Macrophomina phaseolina*	”
34.	”	”	*P. restrictum*	”
35.	”	Leaves	*A. niger*	”
36.	”	”	*P. restrictum, A. niger*	”
37.	”	”	*P. restrictum*	”
38.	”	”	*P. restrictum*	”
39.	”	Roots	*P. restrictum, A. niger*	”
40.	”	”	*P. restrictum, A. niger*	”
41.	”	”	*P. restrictum, A. niger*	”
42.	”	”	*P. restrictum, A. niger*	”
43.	”	Roots	*A. niger, A. flavus*	”
44.	”	Leaves	*P. restrictum, A. niger, A. flvus*	”
45.	”	Roots	*A. niger*	”
46.	”	Leaves	*A. niger, P. restrictum*	”
47.	”	Roots	*A. niger, A. flavus*	”.
48.	*S. persica*	Stem	*A. niger, P. restrictum*	”
49.	”	Roots	*A. niger, A. flavus*	”
50.	”	Stem	*A. niger, A. flavus*	”
51.	”	Stem	*A. niger*	”
52.	*S. oleoides*	Leaves	*A. niger*	”
53.	”	Roots	*A. niger*	”
54.	”	Leaves	*A. niger, A. flavus*	”
55.	*S. persica*	Roots	*A. flavus*	Jamshoro
56.	”	Stem	*A. flavus, P. canescens*	”
57.	”	Roots	*A. niger*	”
58.	*S. oleoides*	Roots	*A. flavus, P. restrictum*	Sanjar
59.	”	Roots	*M. phaseolina*	Sehra
60.	*S. persica*	Stem	*M. phaseolina*	Makle
61.	”	Stem	*A. flavus*	”
62.	*S. oleoides*	Leaves	*A. niger*	Jampeer
63.	”	Roots	*A. niger*	”
64.	”	Leaves	*A. niger*	”
65.	*S. persica*	Roots	*A. flavus*	”
66.	”	Stem	*A. niger*	JangShahi
67.	”	Stem	*A. niger*	”
68.	*S. oleoides*	Leaves	*A. flavus*	”
69.	”	Roots	*A. niger*	Moro
70.	”	Leaves	*A. niger, P. citrinum*	”
71.	”	”	*A. flavus*	”
72.	”	Roots	*A. niger*	Makle
73.	”	Leaves	*P. citrinum*	”
74.	”	Roots	*A. niger, A. flavus*	”

Table 2. Growth inhibition of *Macrophomina phaseolina, Rhizoctonia solani, Fusarium solani* and *F. oxysporum* by endophytic *Penicillium* species isolated from *Salvadora* species in dual culture plate assay.

Fungus No.	*Penicillium* spp.	*M. phaseolina*	*R. solani*	*F. solani*	*F. oxysporum*
		Zone of inhibition (mm)			
EFS-31	*P.restrictum*	7.0	8.0	8.0	0
EFS-38	*P.restrictum*	3.8	3.0	21.0	22
EFS-56	*P.canescens*	2.0	0	0	7.0
EFS-69	*P.citrinum*	5.0	5.0	2.0	8.1

Table 3. *In vitro* growth inhibition of *Macrophomina phaseolina, Rhizoctonia solani, Fusarium solani* and *F. oxysporum* by culture filtrates of endophytic *Penicillium* species isolated from *Salvadora* species.

Fungus No.	*Penicillium* spp.	*M. phaseolina*	*R. solani*	*F. solani*	*F. oxysporum*
		Zone of inhibition (mm)			
	Control	0	0	0	0
	+ ve control (carbendazim 20 µg/disc)	8.0	10.0	8.0	7.0
EFS-31	*P. restrictum*				
	20µl/disc	6.0	3	0	4
	40 µl/disc	7.4	7	5	8
	60 µl/disc	10	8	9	9
EFS-38	*P. restrictum*				
	20µl/disc	0	7	0	5
	40 µl/disc	5	7	4	5
	60 µl/disc	9	8	6	9
EFS-56	*P. canescens*				
	20µl/disc	3	5	5	5
	40 µl/disc	5	8	6	5
	60 µl/disc	8	9	8	6
EFS-69	*P. citrinum*				
	20µl/disc	6	3	0	4
	40 µl/disc	7	7	5	5
	60 µl/disc	9	8	11	6

Table 4. *In vitro* growth inhibition of *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Salmonella typhimurium* by culture filtrates of endophytic *Penicillium* species isolated from *Salvadora* species.

Fungus No.	*Penicillium* spp.	*B. subtilis*	*S. aureus*	*S. typhimurium*	*E. coli*
		Zone of inhibition (mm)			
	Control	0	0	0	0
	+ ve control (streptomycin 20 µg/disc)	14	10.0	8.5	14
EFS-31	*P. restrictum*				
	20µl/disc	0	0	7	0
	40 µl/disc	0	0	7	0
	60 µl/disc	8	11	10	6
EFS-38	*P. restrictum*				
	20µl/disc	0	0	0	0
	40 µl/disc	6	0	0	0
	60 µl/disc	9	7	6	6
EFS-56	*P. canescens*				
	20µl/disc	7	0	0	0
	40 µl/disc	8	7	7	0
	60 µl/disc	10	11	10	8
EFS-69	*P. citrinum*				
	20µl/disc	0	7	7	8
	40 µl/disc	7	9	8	11
	60 µl/disc	12	11	10	13

Discussion

Natural products especially of higher plants and microbial origins have served as rich source of novel drugs. In the past 50 years, number of highly successful drugs based upon fungal metabolites was discovered (Smedsgaard & Nielsen, 2005). Among them, endophytic fungi are also considered as an outstanding source of bioactive compounds due to its ability to occupy any plants at any environments (Strobel & Daisy 2003). In this study, *Aspergillus niger* was found predominant endophytic fungus associated with *Salvadora* spp., followed by *A. flavus. A. niger*, a common contaminant of foods and fruits is also known to occur as endophyte (Raghunath *et al.,* 2012). Liu *et al.,* (2013) has been reported two new 6, 8(14), 22-hexadehydro-5α, 9α-epidioxy-3, 15-dihydroxy sterols, nigerasterols A and B from endophytic *Aspergillus niger.* Similarly, Ravindran *et al.,* (2012) has been reported endophytic association of *A. flavus* with mangroves.

In this study three species of endophytic *Penicillium* isolated from *Salvadora* spp., showed significant antibacterial and antifungal activity against common laboratory bacteria and root rotting fungi. Since the discovery of penicillin a number of drugs have been developed from fungal metabolites. The antitumor antibiotic GKK1032 (GKK1032A1, A2, A3 and B) are manufactured from *Penicillium* species (Koizumi *et al*., 2001). Similarly, a novel antitumor antibiotic, methylenolactocin isolated from culture filtrate of *Penicillium* sp., was also found active against Gram-positive bacteria (Park *et al*., 1988).

El-Neketi *et al.,* (2013) reported five new compounds from endophytic *Penicillium citrinum* isolated from *Ceratonia siliqua.* Similarly Oliveria *et al*., (2009) isolated 8-methoxymellein and 5-hydrooxymellein from endophytic *Penicillium* spp., isolated from leaves of *Alibertia macrophylla.* These compounds were first time isolated from genus *Penicillium.* Vega *et al.,* (2006) reported known and possibly two new species of *Penicillium* from coffee plants. Meng *et al.,* (2011) reported endophytic *P. chrysogenum* as a new source of hypocrellins. Similarly five new picolinic acid derivatives, penicolinates (A-E) together with four known compounds have been reported from endophytic *Penicillium* sp. (Intaraudom *et al.,* 2013).

Species of *Penicillium* are generally considered as soil inhabitant or as contaminant of foods, fruits, fibers and other starchy materials, but these findings indicate endophytic *Penicillium* as a source of new bioactive metabolites, which also play their role in plants against stress tolerance (Khan & Lee, 2013). Secondary metabolites from endophytic fungi particularly *Penicillium* spp., offer an exciting area of research for the discovery of novel compounds for use in medicinal field as well as in agriculture.

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