Pak. J. Bot., 50(4): 1621-1628, 2018.

ROLE OF ENDOPHYTIC *PENICILLIUM* SPECIES IN SUPPRESSING THE ROOT ROTTING FUNGI OF SUNFLOWER

FAIZAH UROOJ[1], HAFIZA FARHAT[1], SYED ABID ALI[2], MARIYAM AHMED[1], VIQAR SULTANA[3], ZAFAR IQBAL SHAMS[4], JEHAN ARA[5] AND SYED EHTESHAMUL-HAQUE[1*]

[1]Agricultural Biotechnology & Phytopathology Laboratory, Department of Botany, University of Karachi, Karachi 75270, Pakistan
[2]HEJ Research Institute of Chemistry, (ICCBS), University of Karachi, Karachi-75270, Pakistan
[3]Department of Biochemistry, University of Karachi, Karachi-75270, Pakistan
[4]Institute of Environmental Studies, University of Karachi, Karachi-75270, Pakistan
[5]Department of Food Science & Technology, University of Karachi, Karachi-75270, Pakistan
[*]Corresponding author's email: sehaq@uok.edu.pk

Abstract

Micro-fungi have been the source of novel and pharmacologically active compounds over decades. Among them, genus *Penicillium* has been recognized as a rich source of bioactive metabolites. In this study, out of 80 plant samples, endophytic *Penicillium* species were isolated from 14 samples (root, stem and leaves) and identified as *P. asperum, P. citrinum, P. duclauxi, P. javanicum, P. lividum, P. nigricans, P. decumbens, P. purpurogenum* (3 isolates), *P. lilacinum, P. restrictum, P. rugulosum* and *P. thomii*. In dual culture plate assay all the fourteen isolates of *Penicillium* inhibited all four test fungi *Fusarium oxysporum, F. solani, Rhizoctonia solani* and *Macrophomina phaseolina* by producing zone of inhibition. Cell free culture filtrate also showed strong antifungal activity at higher dosages. Most of the isolates were significantly suppressed root rotting fungi and improved growth of sunflower in screen house experiments when applied alone or in soil amended with neem (*Azadirachta indica* A.Juss) cake.

Key words: Endophytes, *Penicillium,* Antifungal, Root rotting fungi, Sunflower.

Introduction

Interactions of plants with microorganisms exist as a composite network; some of them may be beneficial, while others are harmful. But the beneficial microorganisms are considerably largest and still broadly unexplored. Some of the beneficial microorganisms, which colonize healthy plant tissue without causing any apparent symptoms of disease, called endophytes (Gherbawy & Gashgari, 2014; Shafique *et al*., 2015; Korejo *et al.,* 2017). They are increasingly gaining scientific and commercial interest because of their potential to improve plant growth through and suppression of plant diseases (Afzal *et al.,* 2013; Khan & Lee, 2013). Endophytes produce several compounds that promote growth of plants and help them adapt better to the environment (Rashid *et al.,* 2012). Endophytes play crucial role in phyto-stimulation, pigment and enzyme production, antimicrobial activity, bioactive and novel compound production, nutrient cycling and bioremediation (Nair & Padmavathy, 2014).

Antifungal and antibacterial activities of plant endophytic fungi have been reported by several researchers (Bhardwaj *et al*., 2015; Gherbawy & Gashgari, 2014). Among the endophytic fungi genus *Penicillium* has been recognized as a rich source of bioactive metabolites (Fill *et al*., 2007). *Penicillium* is one of the largest and most important genera of microscopic fungi, with over 400 described species, distributed worldwide (Visagie *et al*., 2014). *Penicillium* species are generally considered as soil inhabitant or as contaminant of food, fruits, fibers and other starchy materials, which were found to play important role in plants against stress (Ali *et al*., 2011; Khan & Lee, 2013). *Penicillium* species produce a range of medicinally important metabolites including antimicrobial (Lucas *et al*., 2007), antifungal (Nicoletti *et al*., 2007), anti-cancer and insecticidal (Singh, 2003; Stierle *et al*., 2006) and nematicidal (Qureshi *et al*., 2012). In our previous study, we have reported antimicrobial activity of endophytic *Penicillium* species, isolated from *Salvadora* species (Korejo *et al.,* 2014). The present report describes the isolation and identification of endophytic *Penicillium* species from wild and cultivated plants and their biocontrol potential against root rotting fungi infecting sunflower.

Materials and Methods

Collection of plant samples and site: In this study, 80 healthy plant samples (with hypothesis that they harbor unique microbial community) belonging to 29 plant species *viz*., *Abelmoschus esculantus* (L.) Moench, *Achyranthus aspera* L., *Allium cepa* L., *Amaranthus* sp., *Arachis hypogea* L., *Atriplex stocksii* Boiss., *Azadirachta indica* A.Juss., *Brassica campestris* L., *Capsicum annuum* L., *Carica papaya* L., *Chenopodium* sp., *Citrullus lanatus* (Thunb.) Mansf., *Corchorus tridens* L., *Cucurbita pepo* L., *Cyamopsis tetragonoloba* (L.) Taub., *Euphorbia hirta* L., *Helianthus annuus* L., *Lactuca sativa* L., *Luffa aegyptiaca* Mill., *Lycopersicon esculentum* Mill., *Momordica charantia* L., *Pennisetum glaucum* (L.) R.Br., *Solanum melongena* L., *Sorghum bicolor* (L.) Moench, *Spinacea oleracea* L., *Tribulus terristeris* L., *Trigonellafoenum- graecum* L., *Triticum aestivum* L. and *Zea mays* L. were collected from agricultural fields of Malir, Karachi (Memon Goth, Kathor) and Karachi University.

Karachi is located between 24º 45' N to 25º 37' N and 66º 42' E 67º 34 E along the shoreline-of the Arabian Sea. The area is classified as arid hot desert. It is naturally a shrub land. It experiences low average precipitation (25 cm per annum). Its seasonal temperature fluctuates between 13ºC to 36ºC and rarely falls below 9ºC during winters and elevates above 36ºC during summers. Soil in Campus-Area is sandy loam and mostly alkaline in nature (electrical conductivity 0.80-2.80 mS cm^{-1}) with maximum water holding capacity is 23.50–41.00% (Rab *et al*., 2016). Soil characteristics in Memon Goth was similar to Campus, whereas in Kathor, soil contains higher percentage of sands. Isolation of endophytic *Penicillium* was made within 24 hours.

Isolation and identification of endophytic *Penicillium:* One gram of plant samples (roots, stems and leaves) was separately washed under tap water, sterilized with 1% bleach for 3 minutes, then with 70% alcohol for 3 minutes and finally washed with distilled water. Each sample was chopped in sterilized grinder with 50mL sterilized water and dilutions of each sample were made up to $1:10^4$ and 0.1mL suspension from final dilution was transferred onto a Petri-dish containing Potato Dextrose Agar (PDA) supplemented with penicillin (100,000 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), Raper & Thom (1949) and Visagie *et al*., (2014).

***In vitro* dual culture plate assay for determining the antifungal activity of *Penicillium* species:** Antifungal activity of *Penicillium* species was made against four common root rotting fungi *viz*., *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium solani* and *F. oxysporum*. A 5mm agar disc of test *Penicillium* was inoculated on one side of 90mm Petri-dish containing Czapek's Dox Agar, pH7.2. On the other side of same Petri-dish, a 5mm disc of test pathogen was inoculated and incubated at 28°C for 5 days. Zone of inhibition was measured, averaged and expressed in mm (Korejo *et al.,* 2014). There were four replicates of each test and repeated twice.

Preparation of culture filtrates and antifungal activity: Test *Penicillium* species were grown in 250mL conical flask containing 100mL Czapek's Dox broth. Each flask was inoculated with 5mm disc of test *Penicillium*, cut from the margin of vigorously growing culture. The 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 propagules of *Penicillium*, if any. To examine the antifungal activity of secondary metabolites of *Penicillium* species thick sterile filter paper discs were loaded with sterile culture filtrate of each *Penicillium* species at 20, 40 and 60µl/disc and dried. These discs were placed at different places of plates containing Czapek's Dox Agar. In the center of Petri dishes a 5mm disc of test fungus was inoculated. Discs loaded with sterile broth of Czapek's Dox broth were served as control, whereas carbendazim at 20µg/disc were 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).

Effect of endophytic *Penicillium* species on root rotting fungi on sunflower: The experiment was carried out in earthen pots (15cm diam.) in screen house, where neem (*Azadirachta indica* A. Juss) cake (Neemex powder), purchased from Sigma Energy (pvt) Ltd, Karachi, was mixed with sandy loam soil (pH8.0) at 1% w/w and transferred to each pot at 1 Kg per pot. The soil was naturally infested with root rotting fungi *Macrophomina phaseolina* (2-8 sclerotia g^{-1} of soil) as determined by wet sieving and dilution plating (Sheikh & Ghaffar, 1975), 3-10% colonization of *Rhizoctonia solani* on sorghum seeds used as baits (Wilhelm, 1955) and 3000 cfu g^{-1} of soil of a mixed population of *Fusarium oxysporum* and *F. solani* as determined by soil dilution technique (Nash & Snyder, 1962). The pots were watered for 7 days to allow complete decomposition of organic matter. Sunflower (*Helianthus annuus*) seeds were sown in each pot at 6 seeds per pot and 25mL aqueous suspension of *Penicillium* species ($8x10^7$ cfu/mL) grown in Potato Dextrose broth was drenched onto each pot. After germination four seedlings were kept in each pot and excess were removed. In another set, *Penicillium* was inoculated in un-amended soil for comparison. Plants grown in un-amended or un-inoculated soil are served as control, while carbendazim (25mL of 200ppm) are served as positive control. Observations were recorded after 45 days. To assess the efficacy of soil amendment with neem cake and *Penicillium*, plants were uprooted and roots were washed thoroughly with sterilized water and the causal fungi were isolated as described by Mansoor *et al*., (2007). Fungi, which were emerged from root pieces, were identified and infection percentage were calculated. Data on plant growth was also recorded. The experiment was conducted in March 2016 and repeated in March 2017 with similar conditions to confirm the results.

Results

Isolation of endophytic *Penicillium* species and growth inhibition of root rotting fungi by *Penicillium* species in dual culture plate assay: Out of 80 plant samples (roots, stems and leaves) examined, endophytic *Penicillium* were isolated from 14 samples, belonging to 12 species (Table 1). Isolates of *Penicillium* were identified as *P. asperum, P. citrinum, Penicillium* species, *P. duclauxi, P. javanicum, P. lividum, P. nigricans, P. decumbens, P. purpurogenum* (3 isolates), *P. lilacinum, P. restrictum, P. rugulosum* and *P. thomii* (Table 1). All isolates of *Penicillium* were tested against 4 root rotting fungi *M. phaseolina, R. solani, F. solani* and *F. oxysporum in vitro*. In dual culture plate assay, all of the subjected 14 isolates showed antifungal activity by producing zone of inhibition (Table 1). *Penicillium duclauxi* and *P. lividum* produced large sized zone (more than 10mm) against all the four test fungi compared to other isolates.

Table 1. Growth inhibition of *Macrophomina phaseolina*, *Rhizoctonia solani*, *Fusarium solani* and *F. oxysporum* in dual culture plate assay by the endophytic *Penicillium* species isolated from different wild and cultivated plants.

Fungus #	*Penicillium* spp.	Host name	Plant part	*M. phaseolina*	*R. solani*	*F. solani*	*F. oxysporum*
				Zone of inhibition (mm)			
EPSMR1	*P. citrinum*	*Solanum melongena* L. (Solanaceae)	Root	4	4	20	20
EPSMS2	*P. lilacinum*	*Solanum melongena* L. (Solanaceae)	Stem	6	8	11	14
EPSML3	*P. purpurogenum*	*Solanum melongena* L. (Solanaceae)	Leaf	6	5	25	17
EPSLR4	*P. nigricans*	*Lycopersicon esculentum* Mill., (Solanaceae)	Root	5	25	16	21
EPAAR5	*P. rugulosum*	*Achyranthus aspera* L. (Amaranthaceae)	Root	3	12	11	20
EPAIR6	*P. decumbens*	*Azadirachta indica* A.Juss (Meliaceae)	Root	5	25	13	20
EPEHS7	*P. purpurogenum*	*Euhorbia hirta* L. (Euphorbiaceae)	Stem	6	5	25	17
EPCTS8	*P. restrictum*	*Chorchorus tridens* L. (Malvaceae)	Stem	2	2	5	5
EPASS9	*P. duclauxi*	*Atriplex stocksii* (Amaranthaceae)	Stem	18	13	11	14
EPHAL10	*P. asperum*	*Helianthus annuus* L. (Asteraceae)	Leaf	2	2	5	5
EPAER11	*P. thomii*	*Abelmoschus esculentus* L. (Malvaceae)	Root	5	8	5	6
EPMCL12	*P. lividum*	*Momordica charantia* L. (Cucurbitaceae)	Leaf	18	13	11	14
EPSLR13	*P. javanicum*	*Lycopersicon esculentum* Mill., (Solanaceae)	Root	5	24	17	22
EPAER14	*P. purpurogenum*	*Abelmoschus esculentus* L. (Malvaceae)	Root	5	3	21	12

Growth inhibition of root rotting fungi by the cell-free culture filtrates of endophytic *Penicillium* species: Cell-free culture filtrate of *Penicillium* caused growth suppression of root rotting fungi *viz*; *M. phaseolina*, *R. solani*, *F. solani* and *F. oxysporum In vitro* (Table 2). *Macrophomina phaseolina* was inhibited by culture filtrates of *P. lilacinum*, *P. nigricans* and *P. thomii*at 60µl/disc by producing maximum zone of 20 mm. *P. lilacinum*, *P. nigricans* and *P. thomii* also showed zone of inhibition of 15mm at 20µl/disc and 17mm at 40µl/disc. *Rhizoctonia solani* was inhibited by producing zone of 14mm at 60µl/disc from culture filtrates of *P. lilacinum*, *P. purpurogenum* (EPSML3), *P. purpurogenum* (EPEHS7), *P. asperum* and *P. purpurogenum* (EPAER14). *P. nigricans* and *P. thomii* produced zone of inhibition of 17mm at 60µl/disc against *F. solani*. *Penicillium decumbens*, *P. citrinum*, *P. purpurogenum* (EPSML3), *P. regulosum*, *P. purpurogenum* (EPEHS7), *P. duclauxi*, *P. asperum*, *P. thomii*, *P. javanicum* and *P. purpurogenum* (EPAER14) produced zone of inhibition ranging from 12-14mm at 60µl/disc against *F. oxysporum* (Table 2).

Suppression of root rotting fungi of sunflower by the endophytic *Penicillium* species in screen house experiment (2016): Plants that were grown in soil amended with neem (*Azadirachta indica*) cake, generally showed less infection of root rotting fungi compared to plants, which are grown in natural soil (un-amended soil). Most of the plants inoculated with endophytic *Penicillium* species showed less infection of root rotting fungi as compared to untreated control. Plants that were grown in pots received endophytic *P. regulosum* in natural soil and also in amended soil with neem cake showed no infection of *F. oxysporum* (Table 3). Whereas, *P. decumbens*, *P. nigricans*, *P. regulosum*, *P. citrinum*, *P. purpurogenum* (EPSML3), *P. duclauxi*, *P. thomii*, *P. javanicum* and *P. asperum* in amended soil with neem cake also showed no infection of *F. oxysporum*. Combined effect of isolates *P. decumbens*, *P. nigricans*, *P. citrinum*, *P. lilacinum*, *P. purpurogenum* (EPSML3), *P. duclauxi*, *P. lividum*, *P. purpurogenum* (EPEHS7), *P. restrictum*, *P. thomii*, *P. purpurogenum* (EPAER14), *P. javanicum* with neem cake showed no infection on *F. solani*. *P. decumbens*, *P. nigricans*, *P. regulosum* and *P. javanicum* also showed no infection of *F. solani* when used alone. *P. lividum* alone showed no infection of *M. phaseolina* on sunflower roots. Combined effect of *P. decumbens*, *P. nigricans*, *P. regulosum*, *P. thomii* and *P. javanicum* with Neem cake showed significant reduction on infection of *M. phaseolina*. Application of *P. decumbens*, *P. nigricans*, *P. citrinum*, *P. lividum*, *P. purpurogenum* (EPEHS7), *P. purpurogenum* (EPAER14) and *P. javanicum* showed no infection of *R. solani*. *P. decumbens*, *P. regulosum*, *P. citrinum*, *P. lilacinum*, *P. purpurogenum* (EPSML3), *P. duclauxi*, *P. purpurogenum* (EPEHS7), *P. restrictum*, *P. purpurogenum* (EPAER14), *P. javanicum* with neem cake showed no infection of *R. solani*. While *P. nigricans*, *P. lividum*, *P. thomii* and *P. asperum* significantly suppressed the *R. solani* infection when applied in neem cake amended soil (Table 3).

Greater plant height was produced by *P. purpurogenum* (EPEHS7), *P. restrictum*, *P. purpurogenum* (EPAER14) and *P. asperum* when applied in neem cake amended soil. However, the effect of *P. restrictum* and *P. asperum* with neem cake were significant on fresh shoot weight (Table 4). *Penicillium nigricans*, *P. thomii* and *P. javanicum* alone showed significant result on root length and root weight whereas, *P. decumbens* and *P. duclauxi* with neem cake showed greater root length (Table 4).

Suppression of root rotting fungi of sunflower by the endophytic *Penicillium* species in screen house experiment (2017): In the experiment repeated in 2017 generally showed less infection of root rotting fungi in plants, which were grown in soil amended with neem cake compared to plant that were grown in natural soil (un-amended soil). Plants, which were grown in pots, received endophytic *Penicillium* isolates that caused significant reduction of *F. oxysporum* except *P. purpurogenum* (EPSML3) and *P. lividum* (Table 5). Whereas pots received endophytic *P. citrinum*, *P. purpurogenum* (EPSML3), *P. nigricans*, *P. regulosum*, *P. decumbens*, *P. duclauxi*, *P. thomii*, *P. javanicum* showed complete suppression of *F. oxysporum* in neem cake amended soil.

Table 2. *In vitro* growth inhibition of *Macrophomina phaseolina, Rhizoctonia solani, Fusarium solani* and *F. oxysporum* by culture filtrates of endophytic *Penicillium* species isolated from wild and cultivated plant 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 20µg/disc)	8	5	9	7
EPSMR1	*P. citrinum*				
	20 µl/disc	8	8	8	10
	40 µl/disc	8	10	10	10
	60 µl/disc	16	12	10	12
EPSMS2	*P. lilacinum*				
	20 µl/disc	15	10	10	5
	40 µl/disc	17	10	12	5
	60 µl/disc	20	14	12	8
EPSML3	*P. purpurogenum*				
	20 µl/disc	12	8	10	8
	40 µl/disc	14	8	12	8
	60 µl/disc	14	14	14	12
EPSLR4	*P. nigricans*				
	20 µl/disc	15	0	11	8
	40 µl/disc	17	4	15	9
	60 µl/disc	20	8	17	12
EPAAR5	*P. rugulosum*				
	20 µl/disc	11	6	8	9
	40 µl/disc	16	10	8	12
	60 µl/disc	16	12	12	12
EPAIR6	*P. decumbens*				
	20 µl/disc	12	5	14	12
	40 µl/disc	14	8	14	14
	60 µl/disc	14	8	14	14
EPEHS7	*P. purpurogenum*				
	20 µl/disc	12	8	10	8
	40 µl/disc	14	8	12	8
	60 µl/disc	14	14	14	12
EPCTS8	*P. restrictum*				
	20 µl/disc	8	0	8	8
	40 µl/disc	10	5	8	9
	60 µl/disc	11	7	12	11
EPASS9	*P. duclauxi*				
	20 µl/disc	12	0	12	10
	40 µl/disc	16	6	14	10
	60 µl/disc	16	8	14	12
EPHAL10	*P. asperum*				
	20 µl/disc	10	8	12	10
	40 µl/disc	12	10	16	12
	60 µl/disc	12	14	16	12
EPAER11	*P. thomii*				
	20 µl/disc	15	0	11	8
	40 µl/disc	17	4	15	9
	60 µl/disc	20	8	17	12
EPMCL12	*P. lividum*				
	20 µl/disc	12	8	10	9
	40 µl/disc	12	8	12	11
	60 µl/disc	14	12	13	11
EPSLR13	*P. javanicum*				
	20 µl/disc	10	0	8	8
	40 µl/disc	12	5	9	8
	60 µl/disc	14	8	10	12
EPAER14	*P. purpurogenum*				
	20 µl/disc	12	8	10	8
	40 µl/disc	14	8	12	8
	60 µl/disc	14	14	14	12

Table 3. Effect of endophytic *Penicillium* and neem cake on the infection of *Fusarium solani, F.oxysporum, Rhizoctonia solani* and *Macrophomina phaseolina* on sunflower roots in screen house experiment (2016).

Treatments	Code #	Infection %							
		F. oxysporum		*F. solani*		*M. phaseolina*		*R. solani*	
		NS	AS	NS	AS	NS	AS	NS	AS
Control	--	50	18.7	75	25	75	50	18.7	12.5
Carbendazim	--	25	0	31.2	6.2	12.5	25	12.5	0
P. decumbens	EPAIR6	18.7	0	0	0	25	18.7	0	0
P. nigricans	EPSLR4	6.2	0	0	0	37.5	18.7	0	6.2
P. regulosum	EPAAR5	0	0	0	18.7	6.2	18.7	6.2	0
P. citrinum	EPSMR1	37.5	0	25	0	12.5	25	0	0
P. lilacinum	EPSMS2	25	6.2	18.7	0	6.2	50	6.2	0
P. purpurogenum	EPSML3	50	0	12.5	0	6.2	25	6.2	0
P. duclauxi	EPASS9	50	0	6.2	0	31.2	31.2	6.2	0
P. lividum	EPMCL12	50	6.2	50	0	0	50	0	6.2
P. purpurogenum	EPEHS7	37.5	18.7	37.5	0	50	31.2	0	0
P. restrictum	EPCTS8	50	6.2	6.2	0	12.5	43.7	6.2	0
P. thomii	EPAER11	6.2	0	6.2	0	37.5	18.7	6.2	6.2
P. purpurogenum	EPAER14	37.5	18.7	37.5	0	50	31.2	0	0
P. javanicum	EPSLR13	6.2	0	0	0	37.5	18.7	0	0
P. asperum	EPHAL10	12.5	0	25	18.7	37.5	31.2	6.2	6.2
	$LSD_{0.05}$	Treatment=4.65[1]		Pathogen=2.32[2]		Soil Type=1.64[3]			

1. Mean values in the column showing difference greater than LSD value are significantly different at p<0.05
2. Mean values in the row showing difference greater than LSD value are significantly different at p<0.05
3. Mean values in the NS and AS column showing difference greater than LSD value are significantly different at p<0.05
NS= Natural Soil; AS=Amended Soil

Table 4. Effect of endophytic *Penicillium* spp. and neem cake on the growth of sunflower in green house experiment (2016).

Treatments	Code #	Shoot length (cm)		Shoot weight (g)		Root length (cm)		Root weight (g)	
		NS	AS	NS	AS	NS	AS	NS	AS
Control	--	22.7	39.9	2.53	5.35	6.4	11.6	0.64	0.67
Carbendazim	--	25.8	41.8	2.21	4.51	7.4	12.8	0.71	0.62
P. decumbens	EPAIR6	25.4	44.8	2.43	5.12	11.0	14.0	0.77	0.78
P. nigricans	EPSLR4	28.2	44.0	2.77	5.27	12.2	12.1	1.00	0.64
P. regulosum	EPAAR5	25.2	44.0	2.5	4.75	8.6	12.8	0.78	0.62
P. citrinum	EPSMR1	25.9	46.8	2.18	5.1	9.4	8.6	0.72	0.80
P. lilacinum	EPSMS2	22.6	45.8	2.05	5.39	6.3	5.5	0.66	0.57
P. purpurogenum	EPSML3	25.2	40.8	2.15	4.71	9.3	6.8	0.84	0.64
P. duclauxi	EPASS9	25.4	44.8	2.43	5.12	11.0	14.0	0.77	0.78
P. lividum	EPMCL12	22.6	45.8	2.05	5.39	6.3	5.5	0.66	0.57
P. purpurogenum	EPEHS7	23.4	49.3	1.53	5.73	8.8	7.2	0.58	0.74
P. restrictum	EPCTS8	26.1	49.1	2.14	6.78	9.1	7.5	0.69	0.86
P. thomii	EPAER11	28.2	44	2.77	5.27	12.2	12.1	1.00	0.64
P. purpurogenum	EPAER14	23.4	49.3	1.53	5.73	8.8	7.2	0.58	0.74
P. javanicum	EPSLR13	28.2	44	2.77	5.27	12.2	12.1	1.00	0.64
P. asperum	EPHAL10	26.2	49.1	2.14	6.78	9.1	7.5	0.69	0.86
	$LSD_{0.05}$	5.1[1]	7.8[1]	0.79[1]	1.82[1]	2.5[1]	2.8[1]	0.195[1]	0.3[1]

1. Mean values in the column showing difference greater than LSD value are significantly different at p<0.05
NS= Natural Soil; AS= Amended Soil

Combined effect of *P. nigricans*, *P. citrinum*, *P. lilacinum*, *P. lividum*, *P. restrictum*, *P. thomii*, *P. javanicum* and neem cake showed no infection of *F. solani*. *P. decumbens*, *P. nigricans* and *P. javanicum* that also showed complete suppression of infection of *F. solani*. Plant that received *P. lividum*a alone showed no infection of *M. phaseolina* on sunflower roots. Combined effect of all treatments with neem cake showed significant reduction in infection of *M. phaseolina*. Application of *P. decumbens*, *P. citrinum*, *P. lividum*, *P. purpurogenum* (EPEHS7), and *P. regulosum* showed no infection of *R. solani*. *P. decumbens*, *P. nigricans*, *P. citrinum*, *P. purpurogenum* (EPSML3), *P. duclauxi*, *P. purpurogenum* (EPEHS7), *P. restrictum*, *P. purpurogenum* (EPAER14) and *P. javanicum* with neem cake showed complete suppression of *R. solani* (Table 5).

Furthermore, plants which were grown in soil amended with neem cake generally showed greater height compared to plant grown in natural soil (un-amended soil). Most of the plants inoculated with endophytic *Penicillium* species showed larger shoot length compared to untreated control. Greater plant height was produced by *P. lilacinum* when applied in neem cake amended soil (Table 6).

Table 5. Effect of endophytic *Penicillium* and neem cake on the infection of *Fusarium solani, F. oxysporum, Rhizoctonia solani* and *Macrophomina phaseolina*on sunflower roots in green house experiment (2017).

Treatments	Code #	Infection%							
		F. oxysporum		*F. solani*		*M. phaseolina*		*R. solani*	
		NS	AS	NS	AS	NS	AS	NS	AS
Control	--	50	18.7	50	25	75	75	18.7	12.5
Carbendazim	--	12.5	6.2	31.2	6.2	12.5	25	6.2	6.25
P. decumbens	EPAIR6	12.5	0	0	6.2	25	18.7	0	0
P. nigricans	EPSLR4	6.2	0	0	0	31.2	18.7	6.2	0
P. regulosum	EPAAR5	12.5	0	25	6.2	12.5	12.5	0	6.2
P. citrinum	EPSMR1	37.5	0	25	0	12.5	25	0	0
P. lilacinum	EPSMS2	25	6.2	18.7	0	6.2	50	6.2	6.2
P. purpurogenum	EPSML3	50	0	12.5	6.2	6.2	25	6.2	0
P. duclauxi	EPASS9	25	0	6.2	6.2	31.2	18.7	6.2	0
P. lividum	EPMCL12	50	6.2	50	0	0	50	0	6.2
P. purpurogenum	EPEHS7	37.5	18.7	31.2	12.5	50	31	0	0
P. restrictum	EPCTS8	12.5	6.2	6.2	0	12.5	43.7	6.2	0
P. thomii	EPAER11	6.2	0	6.2	0	37.5	18.7	6.2	6.2
P. purpurogenum	EPAER14	37.5	18.7	31.2	12.5	50	31.2	6.2	0
P. javanicum	EPSLR13	6.2	0	0	0	31.2	18.7	6.2	0
P. asperum	EPHAL10	12.5	12.5	25	18.7	31.2	31.2	6.2	6.2
	$LSD_{0.05}$	Treatment=4.45[1]		Pathogen=2.22[2]		Soil Type=1.57[3]			

1. Mean values in the column showing difference greater than LSD value are significantly different at $p<0.05$
2. Mean values in the row showing difference greater than LSD value are significantly different at $p<0.05$
3. Mean values in the NS and AS column showing difference greater than LSD value are significantly different at $p<0.05$

NS= Natural Soil; AS=Amended Soil

Table 6. Effect of endophytic *Penicillium* species and neem cake on the growth of sunflower in green house experiment (2017).

Treatments	Code #	Shoot length (cm)		Shoot weight (g)		Root length (cm)		Root weight (g)	
		NS	AS	NS	AS	NS	AS	NS	AS
Control	--	32.5	38.9	3.78	6.42	5.7	10.3	0.85	1.31
Carbendazim	--	37.8	42.9	4.52	6.07	8.4	10.2	1.24	1.28
P.decumbens	EPAIR6	44.1	62.7	3.86	10.13	7	7.6	0.86	2.13
P.nigricans	EPSLR4	48.3	62.0	4.89	9.53	8.6	6.5	0.96	1.41
P.regulosum	EPAAR5	45.6	64.1	4.72	9.94	6.5	6.6	0.90	1.28
P.citrinum	EPSMR1	38.5	64.4	3.73	14.25	7.5	7.8	0.88	2.26
P.lilacinum	EPSMS2	34.5	65.5	2.06	10.19	7.0	6.4	0.72	1.61
P.purpurogenum	EPSML3	35.4	60.3	2.405	9.09	6.7	5.9	0.91	1.44
P.duclauxi	EPASS9	44.1	62.7	3.86	10.13	7	7.6	0.86	2.13
P.lividum	EPMCL12	34.5	65.5	2.06	10.19	7.0	6.4	0.72	1.61
P.purpurogenum	EPEHS7	38.5	59	2.45	8.86	8.6	11.1	0.83	1.63
P.restrictum	EPCTS8	41.5	50.0	3.62	8.18	6.1	12.7	0.67	1.86
P.thomii	EPAER11	48.3	62.0	4.89	9.53	8.6	6.5	0.96	1.41
P.purpurogenum	EPAER14	38.5	59	2.45	8.86	8.6	11.1	0.83	1.63
P.javanicum	EPSLR13	48.3	62.0	4.89	9.53	8.6	6.5	0.96	1.41
P.asperum	EPHAL10	41.5	50.0	3.62	8.18	6.1	12.7	0.67	1.86
	$LSD_{0.05}$	10.3[1]	8.9[1]	2.27[1]	5.52[1]	3.0[1]	2.1[1]	0.458[1]	1.07[1]

1. Mean values in the column showing difference greater than LSD value are significantly different at $p<0.05$

NS= Natural Soil; AS=Amended Soil

Discussion

Endophytic fungi have proven to be a rich source of novel secondary metabolites with interesting biological activities and a high level of chemical diversity (Schulz & Boyle, 2005). In this study, endophytic *Penicillium* species isolated from cultivated and wild plant species caused growth inhibition of soil borne root rotting fungi *M. phaseolina*, *R. solani*, *F. solani* and *F. oxysporum In vitro*. *Penicillium* are generally considered as soil inhabitant or as contaminant of food, fruits, fibers and other starchy materials, but these findings confirm previous reports about the occurrence of *Penicillium* as endophyte (Korejo *et al.,* 2014; Nicoletti *et al.,* 2014). Furthermore, culture filtrates of these *Penicillium* also showed strong antifungal activity in agar disc diffusion assay, suggesting that endophytic *Penicillium* species as a source of new bioactive metabolites that also play their role in plants against stress tolerance (Khan & Lee, 2013). Our findings are in agreement to Korejo *et al.,* (2014). They reported that culture filtrates of endophytic *Penicillium* species posses significant antifungal activity. Since the discovery of

penicillin a number of drugs have been developed from fungal metabolites including endophytic *Penicillium* species. El-Neketi *et al.,* (2013) has been reported five new compounds from endophytic *Penicillium citrinum*. From endophytic *Penicillium* spp., 8-methoxymellein and 5-hydrooxymellein have been isolated from leaves of *Alibertia macrophylla* (Oliveria *et al.,* 2009). The antifungal activity against phytopathogenic fungi by these isolates suggests that endophytic fungi are producing metabolites that may be toxic or lethal to phytopathogen.

In the present study, biocontrol and plant growth promoting potential of endophytic *Penicillium* was also evaluated in clay pots against root rotting fungi alone or in soil amended with Neem cake using sunflower as test crop. The experiment conducted in 2016 and repeated in 2017 showed significant biocontrol potential of endophytic *Penicillium* against root rotting fungi and improved plant growth. Endophyte protect their host plants by different means, such as the secretion of compounds toxic to pathogens, and occasionally the distraction of the pathogen's cellular membranes, inducing cell-death in the pathogen (Ganley 2008; Prihatini *et al.,* 2016). Several previous research studies have reported the suppression of diseases *via* the inoculation of plants with commonly occurring fungal endophytes (Lee *et al.,* 2009; Poling *et al.,* 2008). There are also reports that endo-symbionts produce plant growth regulators, which enhance the growth of the endophyte infected plants (Khan & Lee, 2013; Rashid *et al.,* 2012). Endophytic *P. funiculosum* LHL06 has also been reported to significantly ameliorated the adverse effects of salinity induced by abiotic stress, and re-programmed soybean to better growth (Khan *et al.,* 2011).

In this study efficacy of *Penicillium* was increased in soil amended with neem cake against root rotting fungi infecting sunflower. Enhancement of biocontrol potential of biocontrol agent in amended soil has been reported (Mansoor *et al.,* 2007). There is also report that *Arabidopsis thaliana* grown in soil amended with barley grain inocula of *P. simplicissimum* GP17-2 or receiving root treatment with its culture filtrate exhibited resistance to *Pseudomonas syringae* pv., syringae (Hossain *et al*., 2007). Neem (*Azadirachta indica*) and its by-products has been broadly reported as a potential fertilizer (Gajalakshmi & Abbasi, 2004) have been reported to control plant diseases caused by fungi and parasitic nematodes (Dubey *et al.,* 2009; Akhtar & Mahmood, 1995). Induction systemic resistance in cotton by the neem cake against soil borne pathogen has been reported by us earlier (Rahman *et al.,* 2016). Thus endophytic *Penicillium* is a source of new bioactive metabolites, which could be exploited in plant protection and also in medicine.

Acknowledgement

Financial assistance provided by the High Education Commission, Islamabad is sincerely acknowledged.

References

Afzal, S., S. Tariq, V. Sultana, J. Ara and S. Ehteshamul-Haque. 2013. Managing the root diseases of okra with endo-root plant growth promoting *Pseudomonas* and *Trichoderma viride* associated with healthy okra roots. *Pak. J. Bot.,* 45: 1455-1460.

Akhtar, M. and I. Mahmood. 1995. Evaluation of a Neem based product against root-knot nematode *Meloidogyne incognita*. Tests of agrochemicals and cultivars, *Suppl. Annal. Appl. Biol*., 16: 6-7.

Ali, A., M.S. Haider, I. Khokhar, U. Bashir, S. Mushtaq and I. Mukhtar. 2011. Antibacterial activity of culture extracts of *Penicillium* species against soil-borne bacteria. *Mycopath*, 9: 17-20.

Barnett, H.L. and B.B. Hunter. 1998. *Illustrated Genera of Imperfect Fungi*. 4th ed. TheAmerican Phytopathological Society. St. Paul. Minnesota. pp. 218.

Bhardwaj, A., D. Sharma, N. Jadon and P.K. Agrawal. 2015. Antimicrobial and phytochemical screening of endophytic fungi isolated from spikes of *Pinus roxburghii*. *Arch. Clin. Microbiol.,* 6: 1-9.

Dubey, R.C., H. Kumar and R.R. Pandey. 2009. Fungitoxic effect of neem extracts on growth and sclerotial survival of *Macrophomina phaseolina In vitro*. *J. Am. Sci.*, 5: 17-24.

El-Neketi, M., W. Ebrahim, W. Lin, S. Gedara, F. Badria, H. A. Saad, D. Lai and P. Proksch. 2013. Alkaloids and polyketides from *Penicillium citrinum*,a endophyte isolated from the Moroccan plant *Ceratonia siliqua*. *J. Nat. Prod.*, 76: 1099-1104.

Fill, T.P., G.K. Pereira, R.M. Santos and E. Rodrigues-Fo. 2007. Four additional meroterpenes produced by *Penicillium* sp., found in association with *Melia azedarach*. Possible biosynthetic intermediates to austin. *Z. Naturforschung B*, 62: 1035-1044.

Gajalakshmi, S. and S.A. Abbasi. 2004. Neem leaves as a source of fertilizercum-pesticide vermicompost. *Bioresource Technol.*, 92: 291-296.

Ganley, R. 2008. Density and diversity of fungal endophytes isolated from needles of Pinus radiata. Client Report, (12925). Forest Bio-security Research Council, New Zealand. pp. 12. www.nzfoa.org.nz/images/ stories/pdfs/ content/beneficial/42803-fbrc_endophyte_ report_ final.pdf.

Gherbawy, Y.A. and R.M. Gashgari. 2014. Molecular characterization of fungal endophytes from *Calotropis procera* plants in Taif region (Saudi Arabia) and their antifungal activities. *Plant Biosystems*, 148: 1085-1092.

Hossain, M.M., F. Sultana, M. Kubota, H. Koyama and M. Hyakumachi. 2007. The plant growth-promoting fungus *Penicillium simplicissimum* GP17-2 induces resistance in *Arabidopsis thaliana* by activation of multiple defense signals. *Plant Cell Physiol.,* 48: 1724-1736.

Khan, A.L. and I.J. Lee. 2013. Endophytic *Penicillium funiculosum* LHL06 secretes gibberellin that reprograms *Glycine max* L. growth during copper stress. *BMC Plant Biol.,* 13: 86.

Khan, A.L., M. Hamayun, Y.H. Kim, S.M. Kang and I.J. Lee. 2011. Ameliorative symbiosis of endophyte (*Penicillium funiculosum* LHL06) under salt stress elevated plant growth of Glycine max L. *Pl. Physiol. Biochem*., 49: 852e-861.

Korejo, F., R. Noreen, S.A. Ali, F. Humayun, A. Rahman, V. Sultana and S. Ehteshamul-Haque. 2017. Evaluation of antibacterial and antifungal potential of endophytic fluorescent *Pseudomonas* associated with *Salvadora persica* and *Salvadora oleoides* decne. *Pak. J.Bot.,* 49: 1995-2004.

Korejo, F., S.A. Ali, H.A. Shafique, V. Sultana, J. Ara and S. Ehteshamul-Haque. 2014. Antifungal and antibacterial activity of endophytic *Penicillium* species isolated from Salvadora species. *Pak. J. Bot.,* 46: 2313-2318.

Lee, K.., J.J. Pan and G. May. 2009. Endophytic *Fusarium verticillioides* reduces disease severity caused by *Ustilago maydis* on maize. *FEMS Microbiol. Lett.*, 299(1): 31-37.

Lucas, E.M., M.C. Castro and J.A. Takahashi. 2007. Antimicrobial properties of sclerotiorin, isochromophilone VI and pencolide, metabolites from a Brazilian cerrado isolate of *Penicillium sclerotiorum* Van Beyma. *Brazi. J. Microbiol.,* 38: 785-789.

Mansoor, F., V. Sultana and S. Ehteshamul-Haque. 2007. Enhancement of biocontrol potential of *Pseudomonas aeruginosa* and *Paecilomyces lilacinus* against root rot of mungbean by a medicinal plant *Launaea nudicaulis* L. *Pak. J. Bot*., 39: 2113-2119

Nair, D.N. and S. Padmavathy. 2014. Impact of endophytic microorganisms on plants, environment and humans. *Sci. World J*., 2014: http://dx.doi.org/10.1155/2014/250693.

Nash, S.M. and W.C. Snyder. 1962. Quantitative estimations by plate counts of propagules of the bean root rot Fusarium in field soils. *Phytopath.*, 52: 567-572.

Nicoletti, R., A. Fiorentino and M. Scognamiglio. 2014. Endophytism of *Penicillium* species in woody plants. *The Open Mycology J.,* 8: 1-26

Nicoletti, R., M.P. Lopez-Gresa. E. Manzo, A. Carella and M.L. Ciavatta. 2007. Production and fungitoxic activity of Sch 642305, a secondary metabolite of *Penicillium canescens. Mycopathologia*, 163: 295-301.

Oliveira, C.M., G.H. Silva, L.O. Regasini, L.M. Zanardi, A.H. Evangelista, M.C.M. Young, V.S. Bolzani and A.R. Araujo. 2009. Bioactive metabolites produced by *Penicillium* sp. 1 and sp. 2, two endophytes associated with *Alibertia macrophylla* (Rubiaceae). *Z. Naturforsch. C,* 64: 824-830.

Poling, S.M., D.T. Wicklow, K.D. Rogers and J.B. Gloer. 2008. *Acremonium zeae* a protective endophyte of maize, produces dihydroresorcylide and 7–hydroxydihydroresorcylides. *J. Agric. Food Chem.*, 56: 3006-3009.

Prihatini, I., M.Glen, T.J. Wardlaw and C.L Mohammed. 2016. Diversity and identification of fungi associated with needles of *Pinus radiate* in Tasmania. *Southern Forests: J. Forest Sci.*, 78: 19-34.

Qureshi, S., K. Ruqqia, V. Sultana, J. Ara and S. Ehteshamul-Haque. 2012. Nematicidal potential of culture filtrates of soil fungi associated with Rhizosphere and rhizoplane of cultivated and wild plants. *Pak. J. Bot.*, 44: 1041-1046.

Qureshi, S.A. 2003. *Studies on Antibiotics from Soil Fungi*. Ph.D. thesis. Department of Biochemistry, University of Karachi, Karachi, Pakistan.

Rab, J.A., M.Z. Iqbal, M. Shafiq and M. Athar. 2016. Studies on vegetation and soil characteristics of Karachi University campus. *Scholar J. Res. Agric. Biol.*, 1: 20-28.

Rahman, A., V. Sultana, J. Ara and S. Ehteshamul-Haque. 2016. Induction of systemic resistance in cotton by the neem cake and *Pseudomonas aeruginosa* under salinity stress and *Macrophomina phaseolina* infection. *Pak. J. Bot*., 48: 1681-1689.

Raper, K.B. and C. Thom. 1949. *A Manual of the Penicillia.* The Williams & Wilkins Company, Baltimore, pp. 875.

Rashid, S., T.C. Charles and B.R. Glick. 2012. Isolation and characterization of new plant growth-promoting bacterial endophytes. *Appl. Soil Ecol.,* 61: 217-224.

Schulz, B. and C. Boyle. 2005. The endophytic continuum. *Mycol. Res.*, 109: 661-686.

Shafique, H.A., R. Noreen, V. Sultana, J. Ara and S. Ehteshamul-Haque. 2015. Effect of endophytic *Pseudomonas aeruginosa* and *Trichoderma harzianum* on soil-borne diseases, mycorrhizae and induction of systemic resistance in okra grown in soil amended with *Vernonia anthelmintica* L. seed's powder. *Pak. J. Bot.*, 47: 2421-2426.

Sheikh, A.H. and A. Ghaffar. 1975. Population study of the sclerotia *of Macrophomina phaseolina* in cotton fields. *Pak. J. Bot.*, 7: 13-17.

Singh, S.B. 2003. Isolation, structure and HIV-1 integrase inhibitory activity of anthoviridicatin E and F, two novel fungal metabolites produced *by Penicillium chrysogenum. Helv. Chem. Acta*, 86: 3380-3385.

Stierle, A.A., D.B. Stierle and K. Kelly. 2006. Berkelic acid, a novel spiroketal with selective anticancer activity from an acid mine waste fungal extremophile. *J. Organic Chem.*, 71: 5357-5360.

Visagie, C.M., J. Houbraken, J.C. Frisvad, S.B. Hong, C.H.W. Klaassen, G. Perrone, K.A. Seifert, J. Varga, T. Yaguchi and R.A. Samson. 2014. Identification and nomenclature of the genus *Penicillium. Stud. Mycol.*, 78: 343-371.

Wilhelm, S. 1955. Verticillium wilt of the strawberry with special reference to resistance. *Phytopath.*, 45: 387-391.

(Received for publication 14 July 2017)