

Bioactivities of endophytic *Penicillia* from Cupressaceae

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Abstract: The cypress family, Cupressaceae, has a global dispersion. Currently, endophytic microorganisms from plants are being investigated for their diversity and bioactivities. Here, we aimed at exploration and characterization of cultivable endophytic fungi from foliar tissues of Cupressaceae, i.e. *Cupressus arizonica*, *C. sempervirens* var. *cereiformis*, *C. sempervirens* var. *fastigiata*, *Juniperus excelsa*, *Juniperus* sp. and *Thuja orientalis*. Asymptomatic fresh foliar tissues, collected from mature healthy plants, were sterilized and the inner layers were plated on culture media at 26-28 °C for 2-12 weeks, until fungal colonies emerged and were purified. Endophytic *Penicillia* i.e. *Penicillium aurantiogriseum*, *P. chrysogenum*, *P. commune*, *P. echinulatum*, *P. expansum* and *P. viridicatum* were the dominant fungi recovered. Results indicated that both host plant and geographical location of sampling affected the biodiversity and bioactivity of endophytic *Penicillia*. Results also indicated that those endophytic *Penicillia* had significant bioactivities. According to our results, both intra- and extra-cellular secondary metabolites from all isolated *Penicillia* had significant cytotoxic and antifungal effects against the model fungus *Pyricularia oryzae* and cypress fungal phytopathogens *Diplodia seriata*, *Phaeobotryon cupressi* and *Spenceriartinsia viticola*. Further studies indicated the significant antimicrobial bioactivities of superior *Penicillia* against model bacteria. Altogether, this study highlights, for the first time, the biodiversity of endophytic *Penicillia* from Cupressaceae plants and documents their significance for agrochemical/drug discovery and for plant disease biocontrol.

Keywords: *Penicillium*, endophyte; *Cupressaceae*, antiproliferative, antifungal, antimicrobial, biocontrol

Introduction

The members of Cupressaceae family (Coniferales) have a worldwide distribution (Fralish and Franklin 2002). These plants are widely used in forestry, horticulture, industry and ethnomedicine and are amongst the most resistant plants to abiotic and biotic stresses (Anonymous, 2004).

The internal tissues of healthy plants are frequently colonized by various microorganisms, termed endophytes (Aly *et al.*, 2010). It has been shown that Cupressaceous plants harbor a vast number of endophytic fungi (Hoffman and Arnold, 2008; Hosseini Moghaddam, 2013). Endophytic microorganisms have revealed a plethora of bioactive natural products which confer major ecological benefits to their host plants (Kusari *et al.*, 2012; Kusari *et al.*, 2013). Moreover, in many cases, plant endophytic fungi secrete the same bioactive metabolites as their host plants (Kusari *et al.*, 2013). Consequently, the secreted secondary metabolites of endophytic

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fungi of Cupressaceae trees may also cause the same bioactivity and associated beneficial health claims of the cypress host plants. Indeed, it has been shown that some endophytic fungi from *Cupressus* and *Juniperus* plant species secrete anti-tumor compounds (Kour *et al.*, 2008, Kumaran *et al.*, 2008; Kusari *et al.*, 2009).

Penicillia are amongst the most prevalent fungi in terrestrial environments. The genus *Penicillium* comprises more than 200 species which produce a vast array of bioactive secondary metabolites (Frisvad and Samson, 2004). It has been also shown that endophytic *Penicillia* are capable of biosynthesizing antifungal metabolites which are active against a number of phytopathogenic fungi (Becker *et al.*, 2012; Nicoletti *et al.*, 2004; Nicoletti *et al.*, 2007; Wang *et al.*, 2007). To our knowledge, only *Penicillium citreoni*, *Penicillium freii* and *Penicillium* sp. are reported as endophytic fungi from Cupressaceae plant family (isolated from *Platycladus orientalis*, Syn. *Thuja orientalis*) and nothing is known about the bioactivities of

such endophytic *Penicillia* (Hoffman and Arnold, 2008; 2010).

There is an ongoing need for novel sources of bioactive metabolites for the treatment of cancer and infectious diseases. Moreover, sustainable forestry demands novel environmentally friendly procedures to protect plants against diseases. Here, the endophytic *Penicillia* of healthy trees of *Cupressus*, *Juniperus* and *Thuja* were explored and further screened for their bioactivities. The potential of such endophytic *Penicillia* for cypress disease biocontrol is also shown, *in vitro*.

Materials and Methods

Sampled sites and Cupressaceae host species

Four locations of Iran, representing the different geographical regions of the country were explored for the plant material collection (Table 1). Plant specimens were collected from *Cupressus semipervirens* var. *cereiformis*, *C. sempervirens* var. *fastigiata*, *C. arizonica*, *Juniperus excelsa* and *Thuja orientalis* (Table 1).

Table 1 Locations, characteristics of sampling sites and endophytic *Penicillia* isolated from aerial parts of healthy cypress host species (2011).

Location (Iran)	Elevation (m)	Host plant species	Plant segment	Isolate	Endophyte Species
Hamedan (Hamedan)	(1900)	<i>Cupressus arizonica</i>	Leaf	CAE ₁	<i>P. expansum</i>
(34.79°N, 48.51°E)		<i>C. arizonica</i>	Stem	CAE ₁₃	<i>P. commune</i>
West of Iran		<i>C. arizonica</i>	Stem	CAE ₁₇	<i>P. echinulatum</i>
		<i>C. arizonica</i>	Stem	CAE ₈₅	<i>P. chrysogenum</i>
		<i>Thuja orientalis</i>	Leaf	POE ₂₈	<i>P. echinulatum</i>
Fars (Shiraz)					
(29.61°N, 52.54°E)	(1486)	<i>C. arizonica</i>	Twig	CAE ₃₉	<i>P. aurantiogriseum</i>
South of Iran		<i>Cupressus sempervirens</i> var. <i>cereiformis</i>	Twig	CSE ₆₅	<i>P. viridicatum</i>
		<i>C. s. var. cereiformis</i>	Twig	CSE ₇₁	<i>P. viridicatum</i>
		<i>C. sempervirens</i> var. <i>fastigiata</i>	Twig	CSE ₇₄	<i>P. viridicatum</i>
		<i>C. s. var. cereiformis</i>	Stem	CSE ₈₈	<i>P. commune</i>
		<i>C. s. var. cereiformis</i>	Twig	CSE ₉₇	<i>P. echinulatum</i>
		<i>T. orientalis</i>	Twig	POE ₃₄	<i>P. expansum</i>
Guilan (Manjil; Astara)	(-27 to 1050)				
(36.32°N, 49.11°E ; 38.41°N, 48.87°E)		<i>Juniperus</i> sp.	Twig	-	
North of Iran					
Markazi (Mahalat)					
(33.91°N, 50.45°E)	(1775)	<i>C. arizonica</i>	Stem	CAE ₁₂	<i>P. commune</i>
Center of Iran		<i>J. excelsa</i>	Twig	-	

Isolation, purification and identification of endophytic fungi

Asymptomatic fresh foliar tissues were collected from mature healthy plants in each locality during June to October, 2011, according to Hoffman and Arnold, 2010. Tissue samples were thoroughly washed, cut into 0.5-1 cm pieces and surface sterilized by dipping in 70% ethanol for 2 min, followed by 4% sodium hypochlorite for 90 sec. and 70% ethanol for 2 min and then rinsed 3 times in sterile distilled water for 1 min each. The inner layers were plated in Petri dishes containing Potato Dextrose Agar (PDA) and/or Water Agar medium. Plates were incubated at 26-28 °C and inspected daily for 2-12 weeks. Hyphal tips, from emerging fungi, were isolated and sub-cultured onto PDA and brought into pure culture.

The identification of the endophytic *Penicillia* was achieved by studying their colony morphology and the mode of spore production on CYA (Czapek Yeast extract Agar; Pitt, 1973), MEA (Malt Extract Agar; Raper and Fenel, 1973) and G25N (25% Glycerol Nitrate Agar; Pitt, 1973). Fungal specimens were stained and studied under microscope, using the identification key of Pitt, 1979.

Bioassays

Model target fungi

Pyricularia oryzae HS-1390 was isolated from leaf lesions of *Oryza sativa* plants (provided by Salar Jamali, Guilan University, Iran). The isolate was initially used as a test model in our antiproliferative and antifungal assays. The cypress fungal phytopathogens, i.e. *Diplodia seriata*, *Phaeobotryon cupressi* and *Spencermartinsia viticola* (Botryosphaerales, Ascomycota; J. Abdollahzadeh, Kurdistan University, Iran; Unpublished) were further employed as target fungi in our antifungal assays.

Intra-and extra-cellular metabolite extraction

The endophytic *Penicillia* were inoculated into Potato Dextrose Broth (PDB) and incubated for 12 days at 28 °C, 120 rpm, under dark

condition. Each individual culture broth was then extracted with methanol.

To obtain extracellular metabolites, the fermentation broth was filtered. The organic solvent methanol was then added to each individual culture broth (1:1). The extract was transferred to 4 °C for 12 hours to remove waxy materials. The organic phase was collected by solvent evaporation at 50 °C. The dried methanol extracts were re-dissolved in double distilled water or DMSO to obtain a final concentration of 250 mg ml⁻¹. Finally, the extracts were passed through a filtration membrane (d = 0.22 µm).

To obtain intracellular metabolites, the mycelial biomasses were harvested, thoroughly washed and macerated in methanol (1:5, 2 days). The mycelia were homogenized thoroughly and the supernatants were treated as explained for the extracellular metabolites. Until used for bioassays, the secondary metabolites were kept at -20 °C.

Antiproliferative and antifungal assays

P. oryzae HS-1390 was initially used to screen endophytic *Penicillia* for their antiproliferative and antifungal metabolites. In the first step, a dual culture of each *Penicillium* species and *P. oryzae* was performed on PDA plates at 28 °C. When the *P. oryzae* in control plate covered the whole petri plate, growth of *P. oryzae* in the presence of each *Penicillium* isolate was compared to that of control plate. Diameter (D) of the inhibition zone (mm) was measured and growth inhibition rate was calculated by the following formula:

Growth inhibition rate (%) = (D control - D treated / D control) × 100%

In the next step, the antiproliferative/cytotoxic bioactivity of both intra- and extra-cellular secondary metabolites from *Penicillia* was examined on the germination of *P. oryzae* conidia, as a model. The *P. oryzae* conidial suspension (4 × 10⁴ ml⁻¹; 50 µl including 0.02% yeast extract) was seeded into each well of a 96 well microtiter plate. The sample extract (50 µl) was added to each well in a serial dilution manner to yield the final concentrations of 250, 125, 62.5, 31.25, 15.62 and 7.81 µg ml⁻¹. The assay plates

were incubated at 27 °C for 16 h. The germination and the size of germ tubes were observed microscopically and compared with control to determine the minimum inhibitory concentration (MIC). The experiments were performed in triplicate and were repeated three times.

Furthermore, the dual culture test for each *Penicillia* and each cypress phytopathogen (*D. seriata*, *P. cupressi* and *S. viticola*) was performed, as described above for anti-*P. oryzae* experiment.

Antimicrobial bioactivity assay

The antimicrobial bioactivities of intra- and extra-cellular metabolites from *Penicillia* were examined on the target plant-associated bacteria, *Pseudomonas syringae*, *Erwinia amylovora* and *Bacillus* sp. (A. Ghasemi, Plant Protection Institute, Tehran, Iran). For this purpose, bacteria were grown to obtain 1×10^6 CFU/ml. Microbroth dilution assays were performed as described for anti-*P. oryzae* assay, but in nutrient broth (NB) medium. The assay plates were incubated at 28 °C for 16 h. The growth of target bacteria was observed and compared with that of control to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). The experiments were performed in triplicate and were repeated three times. Data were obtained and presented as IC₈₀ value which represents the concentration of a metabolite that was required for 80% inhibition *in vitro*.

Statistical analysis

All obtained data were subjected to analysis of variances (ANOVA) and means were compared by least significant differences (LSD) test, using SAS statistical software (Steel *et al.*, 1997). The differences among different treatments were determined at 1% level ($P < 0.01$).

Results

Diversity of endophytic *Penicillia* and their biogeography and host identity

We isolated endophytic *Penicillia* associated with healthy aboveground tissues of five closely related cypress species in the Cupressaceae plant

family. *C. sempervirens* var. *cereiformis* and *C. s.* var. *fastigiata* were native species within their natural ranges. The recovered fungi represented six species, including *Penicillium aurantiogriseum*, *P. chrysogenum*, *P. commune*, *P. echinulatum*, *P. expansum* and *P. viridicatum* (Table 2). As it is shown, diversity of endophytic *Penicillia* differed depending on locality and host plant species. The fungi *P. aurantiogriseum* and *P. chrysogenum* were isolated only from *C. arizonica*, and *P. viridicatum* was isolated only from *C. sempervirens* (Table 2). Diversity of *Penicillia* species recovered from native host *C. s.* var. *cereiformis* in Fars and from non-native host *C. arizonica* in Hamedan were considerable (Table 1). Diversity of *Penicillia* species recovered from the host plant species of *Cupressus* was considerable, compared to *Thuja* and *Juniperus* species (Table 1). *P. chrysogenum* was recovered only from Hamedan and *P. aurantiogriseum* and *P. viridicatum* were recovered only from Fars, but *P. commune*, *P. echinulatum* and *P. expansum* were recovered from, at least, two different provinces (Table 1).

Antiproliferative bioactivity of intra- and extra-cellular metabolites of the endophytic *Penicillia*

P. oryzae has served as a model for the primary screening of antiproliferative and antifungal agents (Kobayashi *et al.*, 1996). Accordingly, the bioassay was used to evaluate antiproliferative and growth inhibition activities of both intra- and extra-cellular *Penicillia* metabolites. The results, shown in Tables 3 and 4, indicate that all endophytic *Penicillia* exhibited significant bioactivities.

As indicated in Table 3, the extra-cellular metabolites from endophytic *P. viridicatum* (isolates CSE₇₄ and CSE₇₁), *P. commune* (isolates CSE₈₈ and CAE₁₂), *P. expansum* (isolates CAE₁ and POE₃₄) and *P. chrysogenum* (isolate CAE₈₅) showed, *in vitro*, cytotoxicity effects against *P. oryzae*. The isolate *P. viridicatum* CSE₇₄ showed the most significant bioactivity among all isolates. The extracellular metabolites of this isolate showed cytotoxic effects at 62.5 µg ml⁻¹ and the growth inhibition at 15.6 µg ml⁻¹.

Table 2 Identity of endophytic *Penicillium* species hosted by Cupressaceae plants (2011).

Endophyte species	Isolate (s)	Host plant species	Plant segment	Location (Iran)
<i>P. aurantiogriseum</i>	CAE ₃₉	<i>C. arizonica</i>	Twig	Fars
<i>P. chrysogenum</i>	CAE ₈₅	<i>C. arizonica</i>	Stem	Hamedan
<i>P. commune</i>	CAE ₁₃	<i>C. arizonica</i>	Stem	Hamedan
	CSE ₈₈	<i>C. s. var. cereiformis</i>	Stem	Fars
	CAE ₁₂	<i>C. arizonica</i>	Stem	Markazi
<i>P. echinulatum</i>	CAE ₁₇	<i>C. arizonica</i>	Stem	Hamedan
	POE ₂₈	<i>T. orientalis</i>	Leaf	Hamedan
	CSE ₉₇	<i>C. s. var. cereiformis</i>	Twig	Fars
<i>P. expansum</i>	POE ₃₄	<i>T. orientalis</i>	Twig	Fars
	CAE ₁	<i>C. arizonica</i>	Leaf	Hamedan
<i>P. viridicatum</i>	CSE ₆₅	<i>C. s. var. cereiformis</i>	Twig	Fars
	CSE ₇₄	<i>C. s. var. fastigiata</i>	Twig	Fars
	CSE ₇₁	<i>C. s. var. cereiformis</i>	Twig	Fars

Table 3 The antiproliferative activity of extracellular metabolites from endophytic *Penicillia* on the conidia of *Pyricularia oryzae*.

Isolate	Species	The final concentrations of extracellular metabolites ($\mu\text{g ml}^{-1}$)					
		250.0	125.0	62.5	31.2	15.6	7.8
CSE ₇₄	<i>P. viridicatum</i>	*	*	*	+++	++	-
CSE ₇₁	<i>P. viridicatum</i>	*	*	+++	++	++	+
CSE ₈₈	<i>P. commune</i>	*	*	+++	++	+	+
CAE ₁₂	<i>P. commune</i>	*	+++	+++	++	+	-
CAE ₁	<i>P. expansum</i>	*	+++	+++	++	+	-
POE ₃₄	<i>P. expansum</i>	*	+++	++	++	-	-
CAE ₈₅	<i>P. chrysogenum</i>	*	+++	++	++	-	-
CAE ₃₉	<i>P. aurantiogriseum</i>	+++	+++	++	+	-	-
CAE ₁₇	<i>P. echinulatum</i>	+++	+++	++	-	-	-
POE ₂₈	<i>P. echinulatum</i>	+++	++	++	-	-	-
CAE ₁₃	<i>P. commune</i>	+++	++	-	-	-	-
CSE ₉₇	<i>P. echinulatum</i>	++	++	-	-	-	-
CSE ₆₅	<i>P. viridicatum</i>	+	+	-	-	-	-

Symbols: (*) The *P. oryzae* conidial germination was inhibited; (+++) strong growth inhibition of germ tube ($\leq 1/3$ of control); (++) moderate growth inhibition of germ tube ($1/3$ - $2/3$ of control); (+) low growth inhibition of germ tube ($\geq 2/3$ but less than control); (-) no inhibition (same as control). The observations are averages of 4-6 assays.

Moreover, the intra-cellular metabolites from *P. viridicatum* (isolates CSE₇₄ and CSE₇₁), *P. aurantiogriseum* (isolate CAE₃₉), *P.*

commune (isolates CSE₈₈ and CAE₁₂) and *P. expansum* (isolates CAE₃₄ and POE₁) showed, *in vitro*, cytotoxicity effects against *P. oryzae*

(Table 4). The isolate *P. viridicatum* CSE₇₄ showed the most significant bioactivity among all *Penicillia*. Intracellular metabolites of *P. viridicatum* CSE₇₄ showed cytotoxic effect at 31.2 µg ml⁻¹ and the growth inhibition at 7.8 µg ml⁻¹, which were stronger than those of its extracellular metabolites. The cytotoxic effect of intracellular metabolite from *P. viridicatum* CSE₇₁ was stronger than that of its extracellular metabolite and comparable to that of extracellular metabolite of *P. viridicatum* CSE₇₄. Remarkably, *P. aurantiogriseum* CAE₃₉, which didn't show cytotoxicity by its extracellular metabolite at high concentration of 250.0 µg/ml, exhibited cytotoxicity by its intracellular metabolite at this range. However, the intracellular metabolites of both *P. commune* CSE₈₈ and *P. chrysogenum* CAE₈₅ showed less cytotoxic effects compared to their extracellular metabolites. Surprisingly, *P. viridicatum* CSE₆₅, which was isolated from the same host species as isolate CSE₇₁, exhibited the least cytotoxic and growth inhibition effects on *P. oryzae* among all *Penicillia* (Tables 3 and 4).

Antifungal activity of the endophytic *Penicillia*

The initial antifungal assays of the endophytic *Penicillia* against *P. oryzae* indicated that all *Penicillium* species inhibited the mycelial growth of this fungus, *in vitro*. In this respect, *P. viridicatum* CSE₇₄ was superior to others (Table 5). *P. viridicatum* CSE₇₁, *P. viridicatum* CSE₆₅, *P. aurantiogriseum* CAE₃₉ and *P. commune* CAE₁₂ were also ranked among the most effective isolates.

Furthermore, all *Penicillia* exerted antifungal effects against phytopathogenic fungi of cypress, i.e., *D. seriata*, *P. cupressi* and *S. viticola* (Table 6). According to the results, it seems that the *Penicillia* were more effective on *S. viticola* and on *P. cupressi* than on *D. seriata*. The endophytic *P. viridicatum* CSE₇₄, *P. viridicatum* CSE₇₁ and surprisingly *P. commune* CAE₁₃ had the highest effects on *D. seriata*. The endophytic *P. viridicatum* CSE₇₁ was the second isolate showing a significant antifungal effect on *S. viticola*. Meanwhile, *P. viridicatum* CSE₇₁, *P. echinulatum* CSE₁₇ and *P. chrysogenum* CAE₈ showed similar antifungal effects on *P. cupressi*.

Table 4 The antiproliferative activity of intracellular metabolites from endophytic *Penicillia* on the conidia of *Pyricularia oryzae*.

Isolate	Species	The final concentrations of intracellular metabolites (µg ml ⁻¹)					
		250.0	125.0	62.5	31.2	15.6	7.8
CSE ₇₄	<i>P. viridicatum</i>	*	*	*	*	+++	++
CSE ₇₁	<i>P. viridicatum</i>	*	*	*	+++	+++	++
CAE ₃₉	<i>P. aurantiogriseum</i>	*	+++	+++	++	-	-
CSE ₈₈	<i>P. commune</i>	*	+++	+++	+	-	-
CAE ₁₂	<i>P. commune</i>	*	+++	++	+	-	-
POE ₃₄	<i>P. expansum</i>	*	+++	+	+	-	-
CAE ₁	<i>P. expansum</i>	*	++	+	-	-	-
CAE ₁₃	<i>P. commune</i>	+++	+++	++	+	-	-
CAE ₈₅	<i>P. chrysogenum</i>	+++	+++	++	-	-	-
CAE ₁₇	<i>P. echinulatum</i>	+++	++	+	-	-	-
CSE ₉₇	<i>P. echinulatum</i>	++	++	+	-	-	-
POE ₂₈	<i>P. echinulatum</i>	++	+	-	-	-	-
CSE ₆₅	<i>P. viridicatum</i>	+	+	+	-	-	-

Symbols: (*) The *P. oryzae* conidial germination was inhibited; (+++) strong growth inhibition of germ tube ($\leq 1/3$ of control); (++) moderate growth inhibition of germ tube ($1/3$ - $2/3$ of control); (+) low growth inhibition of germ tube ($\geq 2/3$ but less than control); (-) no inhibition (same as control). The observations are averages of 4-6 assays.

Table 5 The antifungal activity of endophytic *Penicillia* against *Pyricularia oryzae*.

Isolate	Endophytic <i>Penicillia</i>	Radius of mycelial growth (mm)	Growth inhibition of <i>P. oryzae</i> (%)
CSE ₇₄	<i>P. viridicatum</i>	16.6 ± 0.6 ^a	63
CSE ₇₁	<i>P. viridicatum</i>	17.3 ± 1.0 ^{ab}	61
CAE ₃₉	<i>P. aurantiogriseum</i>	17.3 ± 0.6 ^{ab}	61
CSE ₆₅	<i>P. viridicatum</i>	17.6 ± 1.5 ^{ab}	60
CAE ₁₂	<i>P. commune</i>	18 ± 2.0 ^{ab}	60
POE ₃₄	<i>P. expansum</i>	18.3 ± 1.5 ^{abc}	59
CSE ₉₇	<i>P. echinulatum</i>	18.3 ± 2.1 ^{abc}	59
CAE ₁₇	<i>P. echinulatum</i>	18.6 ± 1.1 ^{abcd}	58
CAE ₁	<i>P. expansum</i>	19 ± 1.0 ^{bcd}	57
CAE ₁₃	<i>P. commune</i>	19 ± 1.0 ^{bcd}	57
CSE ₈₈	<i>P. commune</i>	20.6 ± 0.6 ^d	54
POE ₂₈	<i>P. echinulatum</i>	20.3 ± 0.6 ^d	54
CAE ₈₅	<i>P. chrysogenum</i>	21.6 ± 1.5 ^d	52
Control	-	45.0 ± 0.0	0

Data (significant at $P \leq 0.01$) are averages (\pm standard deviation) of three replicates. Similar letters indicate no significant difference.

Table 6 The antifungal activity of endophytic *Penicillia* against cypress phytopathogenic fungi.

Isolate	Endophytic <i>Penicillia</i>	Cypress phytopathogenic fungi		
		Radius of mycelial growth (mm) and growth inhibition (%)		
		<i>S. viticola</i>	<i>P. cupressi</i>	<i>D. seriata</i>
CSE ₇₄	<i>P. viridicatum</i>	12.3 ± 0.6 (71) ^a	14.3 ± 1.1 (67) ^a	15.3 ± 1.1 (65) ^a
CSE ₇₁	<i>P. viridicatum</i>	13.6 ± 1.1 (68) ^{ab}	15.6 ± 0.6 (64) ^{ab}	16.0 ± 0.0 (63) ^a
CAE ₁₇	<i>P. echinulatum</i>	14 ± 1.0 (68) ^b	15.7 ± 0.6 (64) ^{ab}	19.3 ± 1.1 (56) ^{bc}
CSE ₆₅	<i>P. viridicatum</i>	14.3 ± 1.5 (67) ^{bc}	16.3 ± 0.6 (62) ^{bc}	19.6 ± 1.5 (55) ^{bc}
CAE ₈₅	<i>P. chrysogenum</i>	14.6 ± 0.6 (66) ^{bc}	15.6 ± 1.0 (64) ^{ab}	20.3 ± 0.6 (53) ^{bcd}
CSE ₉₇	<i>P. echinulatum</i>	15.6 ± 0.6 (64) ^{cd}	16.3 ± 0.6 (62) ^{bc}	20.3 ± 0.6 (53) ^{bcd}
CAE ₃₉	<i>P. aurantiogriseum</i>	15.6 ± 1.1 (64) ^{cd}	17.3 ± 1.5 (60) ^{bc}	20.6 ± 0.6 (53) ^{cd}
CSE ₈₈	<i>P. commune</i>	15.6 ± 0.6 (64) ^{cd}	17.7 ± 1.0 (59) ^{cd}	21.3 ± 0.6 (51) ^d
CAE ₁₃	<i>P. commune</i>	15.6 ± 0.6 (64) ^{cd}	19.3 ± 1.1 (55) ^{de}	16.6 ± 1.1 (62) ^a
CAE ₁₂	<i>P. commune</i>	17 ± 0.0 (61) ^{de}	17.3 ± 1.1 (60) ^{bc}	19.3 ± 0.6 (56) ^{bc}
POE ₂₈	<i>P. echinulatum</i>	17 ± 1.0 (61) ^{de}	19.3 ± 1.1 (55) ^{de}	22.3 ± 0.6 (49) ^e
CAE ₁	<i>P. expansum</i>	17.3 ± 0.6 (60) ^e	17.6 ± 2.1 (59) ^{cd}	19.0 ± 1.7 (56) ^b
POE ₃₄	<i>P. expansum</i>	16.3 ± 0.6 (52) ^{de}	20 ± 1.0 (54) ^e	20.6 ± 0.6 (53) ^{cd}
Control	-	43.6 ± 1.1 (0)	43.6 ± 1.1 (0)	44.0 ± 1.0 (0)

Data (significant at $P \leq 0.01$) are averages (\pm standard deviation) of three replicates. Similar letters indicate no significant difference. Figures in parentheses are pathogen growth inhibition (%).

Antimicrobial activities of intra- and extra-cellular metabolites of the endophytic *Penicillia* Metabolites extracted from the superior *Penicillia* isolates (*P. viridicatum* CSE₇₄ and *P. viridicatum* CSE₇₁) were further applied on the model bacteria. As indicated in Table 7, all metabolites from both *Penicillium* isolates showed antimicrobial activities against all three bacterial targets. Here, it seemed that the

extracellular metabolites showed more bioactivities than the intracellular ones. The metabolites of *P. viridicatum* CSE₇₄ were more effective against gram-negative bacteria. In general, *P. syringae* bacterium appeared to be more sensitive to *Penicillia*'s metabolites than *E. amylovora* and *Bacillus* sp.

Table 7 The antimicrobial activities of extra- and intra-cellular metabolites from endophytic *Penicillia*.

Fungal isolate	Target bacteria	Extract concentration ($\mu\text{g mL}^{-1}$)			
		MIC ^a		MBC ^b	
		Extracellular	Intracellular	Extracellular	Intracellular
<i>P. viridicatum</i> CSE ₇₄	<i>Pseudomonas syringae</i>	15.6	31.2	62.5	62.5
	<i>Erwinia amylovora</i>	15.6	31.2	31.2	62.5
	<i>Bacillus</i> sp.	31.2	62.5	62.5	125
<i>P. viridicatum</i> CSE ₇₁	<i>Pseudomonas syringae</i>	15.6	31.2	62.5	62.5
	<i>Erwinia amylovora</i>	31.2	62.5	62.5	125
	<i>Bacillus</i> sp.	31.2	62.5	62.5	125

a. Minimum inhibitory concentration

b. Minimum bactericidal concentration.

Data (significant at $P \leq 0.01$) were obtained from three replicates.

Data are reported as IC₈₀ values.

Discussion

Despite their longevity, the members of Cupressaceae are significantly resistant to biotic and abiotic stresses. The only report on ecology of endophytic fungi from Cupressaceous trees, involving *J. virginiana*, *C. arizonica* and *P. orientalis*, suggests that at least 35 endophytic fungal species associate with these plants (Hoffman and Arnold, 2008). However, the bioactivities of such endophytic fungi have not been explored yet.

Here, we aimed at isolation and characterization of cultivable endophytic fungi from Cupressaceae plant species, including *C. sempervirens* var. *fastigiata*, *C. s.* var. *cereiformis* (indigenous to Iran), *C. arizonica*, *Thuja oreintalis* and *Juniperus excelsa* (non-indigenous). We showed in this study that both host plant species and geographical locations of sampling affected the biodiversity of endophytic fungi. It seems from our findings

that a number of different *Penicillium* fungal species could adapt an endophytic lifestyle in Cupressaceous host plants, like *Cupressus* and *Thuja* plant species. Moreover, some *Penicillia* were restricted only to one host, yet others were recovered from more than one host. These findings are in agreement with the former report (Hoffman and Arnold, 2008).

Penicillia have long been well-known for the production of bioactive secondary metabolites (Fleming, 1929). Recent studies have indicated that *Penicillia* can reside inside healthy plants as endophytes. Such endophytic *Penicillia* have shown a vast range of bioactivities (Waqas *et al.*, 2012; Zheng *et al.*, 2012). The beneficial effects of such bioactivities on their host plants are not fully understood yet.

In earlier studies *Penicillium citreoni* and *P. freii* have been isolated, as endophytic fungi, from the cypress *Platyclusus oreintalis* (Syn. *T. orientalis*) (Hoffman & Arnold, 2008;

Hoffman & Arnold, 2010). In this study, for the first time, we showed that at least six species of *Penicillium*, were endophytically associated with healthy plants of Cupressaceae family. The endophytic association of *Penicillium aurantiogriseum*, *P. chrysogenum*, *P. commune*, and *P. expansum* with other plant hosts has already been shown globally (Botella and Diez, 2011; Chlebicky, 2009; Devi et al., 2012; Lu et al., 2010; Meng et al., 2011; Xuan et al., 2010; Xu et al., 2008; Yan et al., 2010). However, this is the first report of endophytic association of *Penicillium aurantiogriseum*, *P. chrysogenum*, *P. commune*, *P. echinulatum*, *P. expansum* and *P. viridicatum* with the members of Cupressaceae plant family. Moreover, *P. echinulatum* and *P. viridicatum* have not previously been reported as endophytic fungi. Also, except for *Penicillium commune* which was new to the mycoflora of Iran, the other *Penicillia* recovered in our survey, have been formerly isolated from soil, fruit and nuts in Iran, as saprophytic, epiphytic or phytopathogenic, but not endophytic, fungi (Abbasi and Aliabadi, 2009; Ershad, 2009). Our findings further indicated that those endophytic *Penicillia* had significant antiproliferative, antifungal and antimicrobial capacities. Using *P. oryzae* as a model we showed that metabolites from all *Penicillium* species had significant cytotoxicity effects. *P. viridicatum* CSE₇₄ and CSE₇₁ showed the most significant antiproliferative bioactivities. Moreover, the intracellular metabolites of the most effective *Penicillia* exert their bioactivities at lower concentrations than the extra-cellular ones. The future metabolomics analysis could unravel the molecules involved, and provide an explanation. In addition, all *Penicillia* species showed significant antifungal effects against three of the cypress phytopathogenic fungi. According to our results, *P. viridicatum* CSE₇₄ was significantly superior to the other species. The fungi *P. viridicatum* CSE₇₄, *P. viridicatum* CSE₇₁ and *P. commune* CAE₁₃ showed the highest antagonistic effects on *D. seriata*. The fungi *P. viridicatum* CSE₇₁, *P. echinulatum* CSE₁₇ and

P. chrysogenum CAE₈ showed similar significant effects on *P. cupressi*. Interestingly, *P. viridicatum* CSE₆₅ whose metabolites showed the least cytotoxic effects on *P. oryzae*, ranked among the most effective species inhibiting the growth of cypress fungal phytopathogens. This finding may suggest that other metabolites like volatile compounds (VOC) might have been involved in such growth inhibition. Alternatively, a combination of VOC and extracellular metabolites may synergistically exert such effect (Strobel et al., 2011).

Our survey on antimicrobial activities of metabolites from *P. viridicatum* CSE₇₄ and *P. viridicatum* CSE₇₄, indicated a broader bioactivity for the cypress endophytic *Penicillia*. Indeed, both isolates inhibited bacterial growth and showed cytotoxic activities on bacterial cells. However, in this case, in comparison with their antiproliferative bioactivities, it seems that extracellular metabolites showed more bioactivities than intracellular ones and that the metabolites were more effective on gram-negative bacteria than on gram-positive ones. This may indicate the significance of *Penicillia*'s secreted metabolites for surviving in niches occupied by bacteria.

The fungus *P. viridicatum* has actually been reported as a corn storage mold and an allergenic, nephrogenic, mycotoxin producer fungus which has also been isolated from mosquito (Cabañes et al., 2010). Our results further support the cytotoxic and antibiotic properties of the identified endophytic *Penicillium* species. However, these findings may indicate a host protective role for endophytic *Penicillia* against biotic and abiotic stresses. Moreover, the most effective metabolites have been obtained from the *Penicillium* isolates recovered from indigenous *C. s.* var. *cereiformis* (Sarve Naz) and *C. s.* var. *fastigiata* (Sarve Shiraz). This may suggest that these endemic plant hosts are evolutionarily dependent on such associations to resist stresses. Hence, the possibility of using such endophytic microorganisms for biocontrol of cypress diseases is intriguing.

However, for such studies, the complex triangle interactions of plant host-endophytic fungi-phytopathogen must be considered (Kusari *et al.*, 2013).

Moreover, the bioactivities of secondary metabolites from *Penicillia* are also dependent on the kind of target microorganism. In our survey, it was clear that *S. viticola* was more sensitive than *P. cupressi* to secondary metabolites from *Penicillia* and they both were more sensitive than *D. seriata* to those metabolites, *in vitro*. Also, *P. syringae* showed more sensitivity to *Penicillia*'s metabolites than *E. amylovora* and *Bacillus* sp., *in vitro*. However, *in vivo* studies are still needed to interpret the real impact of such *Penicillia*'s metabolites on the target microorganisms.

In conclusion, the members of cypress family host a range of endophytic ascomycetous fungi. The results of our study are in agreement with the findings of former studies, in that *Penicillia* are among the prominent members of the ascomycetous fungi. The diversity of novel endophytic *Penicillia* from cypress family in our study and the bioactivities of their secondary metabolites, particularly against cypress fungal phytopathogens, encourages profound research on bioactive endophytic fungi. To our knowledge, this is the first report of the *Penicillia* species living as endophytes inside the *Cupressus arizonica*, *C. s.* var. *cereiformis*, *C. s.* var. *fastigiata* and *Thuja orientalis* and the bioactivities thereof. Those *Penicillia* could potentially serve as biocontrol agents and further as lead isolates for novel agrochemical/drug discovery to combat microbial infectious and probably cancer diseases.

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فعالیت‌های زیستی پنی‌سیلیوم‌های اندوفیت گیاهان خانواده‌ی سرو

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چکیده: گیاهان خانواده سرو گسترشی جهانی داشته و برخی گونه‌ها، زیرگونه‌ها و ارقام آن بومی ایران می‌باشند. در حال حاضر اندوفیت‌های گیاهی به‌سبب تنوع و طیف وسیع اثرات زیستی‌شان تحت بررسی‌های فراوانی هستند. هدف از پژوهش حاضر جداسازی و بررسی اثرات زیستی اندوفیت‌های قارچی اندام‌های هوایی سالم گیاهان خانواده‌ی سرو موجود در ایران بود. بدین‌منظور در طی فصل بهار، تابستان و پاییز سال ۱۳۹۰ خورشیدی (۲۰۱۱ میلادی) به‌صورت تصادفی از اندام‌های سالم درختان سروناز (*C. sempervirens* var. *cereiformis*)، سرو شیراز (*C. sempervirens* var. *fastigiata*)، سرو نقره‌ای (*Cupressus arizonica*)، سرو خمره‌ای (*Thuja orientalis*) و سرو کوهی (*Juniperus excelsa*) در رویشگاه‌های طبیعی استان‌های فارس، گیلان، مرکزی و همدان نمونه‌برداری انجام شد. نمونه‌ها پس از انتقال به آزمایشگاه کشت داده شدند و قارچ‌های جداسازی شده، خالص گردیدند. نتایج نشان داد که گونه‌های جنس پنی‌سیلیوم شامل *Penicillium aurantiogriseum*, *P. chrysogenum*, *P. commune*, *P. echinulatum*, *P. expansum*, *P. viridicatum*, دارای بیشترین فراوانی در بین تمامی گونه‌های جداسازی شده بودند. نتایج حاکی از آن بود که تنوع گونه‌های اندوفیت و فعالیت زیستی‌شان هم به‌گونه‌ی گیاه میزبان و هم به ناحیه‌ی نمونه برداری بستگی داشت. بررسی‌های بیشتر نشان داد که گونه‌های پنی‌سیلیوم جداسازی شده دارای اثرات زیستی قابل‌توجهی بودند. بدین‌منظور، استخراج متابولیت‌های ثانویه درون سلولی و برون سلولی انجام گرفت. سپس اثر ضد جواهره‌زنی کنیدیوم و اثر ضد قارچی متابولیت‌های گونه‌های پنی‌سیلیوم روی قارچ مدل *Pyricularia oryzae* آزمایش شد. جهت بررسی توانایی بیوکنترلی اندوفیت‌ها، از کشت دوطرفه‌ی ایزوله‌های اندوفیت قارچی با سه قارچ بیماریزای درختان سرو شامل *Diplodia seriata*, *Phaeobotryon cupressi* و *Spenceriartinsia viticola* استفاده شد. نتایج نشان داد که تمامی جدایه‌های اندوفیت پنی‌سیلیوم سطوحی از اثرات بازدارندگی را در سطح آزمایشگاهی نشان دادند. همچنین اثرات ضدباکتریایی متابولیت‌های ثانویه درون سلولی و برون سلولی قارچ‌های اندوفیت پنی‌سیلیوم بر روی باکتری‌های گیاهی نشان داده شد. با درنظرگرفتن یافته‌های این پژوهش، به‌نظر می‌رسد که گیاهان سرو ایران میزبان طیف متنوعی از قارچ‌های اندوفیت دارای اثرات زیستی مهم برای کشاورزی و داروسازی می‌باشند.

واژگان کلیدی: پنی‌سیلیوم، اندوفیت، تیره سرو، اثرات ضدقارچی، اثرات ضدباکتریایی، اثرات ضدجواهره‌زنی کنیدیوم، بیوکنترل