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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 *Spencermartinsia 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; Hosseyni 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 oreintalis*, Syn. *Thuja oreintalis*) 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)	*Cupressus arizonica*	Leaf	CAE_{1}	*P. expansum*
(34.79ºN, 48.51ºE)		*C. arizonica*	Stem	CAE_{13}	*P. commune*
West of Iran		*C. arizonica*	Stem	CAE_{17}	*P. echinulatum*
		C. arizonica	Stem	CAE_{85}	*P. chrysogenum*
		Thuja orientalis	Leaf	POE_{28}	*P. echinulatum*
Fars (Shiraz)					
(29.61ºN, 52.54ºE)	(1486)	*C. arizonica*	Twig	CAE_{39}	*P. aurantiogriseum*
South of Iran		*Cupressus sempervirens* var. cereiformis	Twig	CSE_{65}	*P. viridicatum*
		C. s. var. cereiformis	Twig	CSE_{71}	*P. viridicatum*
		C. sempervirens var. fastigiata	Twig	CSE_{74}	*P. viridicatum*
		C. s. var. cereiformis	Stem	CSE_{88}	*P. commune*
		C. s. var. cereiformis	Twig	CSE_{97}	*P. echinulatum*
		T. orientalis	Twig	POE_{34}	*P. expansum*
Guilan (Manjil; Astara) (36.32ºN, 49.11ºE ; 38.41ºN, 48.87ºE) North of Iran	(-27 to 1050)	*Juniperus* sp.	Twig	-	
Markazi (Mahalat)					
(33.91ºN, 50.45ºE)	(1775)	*C. arizonica*	Stem	CAE_{12}	*P. commune*
Center of Iran		*J. excelsa*	Twig	-	

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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* (Botryosphaeriales, 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 ºC, 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 ºC for 12 hours to remove waxy materials. The organic phase was collected by solvent evaporation at 50 ºC. 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 ºC.

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 ºC. 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 (4×10^4 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

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were incubated at 27 ºC 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 ºC 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 IC_{80} 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 CSE_{74} and CSE_{71}), *P. commune* (isolates CSE_{88} and CAE_{12}), *P. expansum* (isolates CAE_1 and POE_{34}) and *P. chrysogenum* (isolate CAE_{85}) showed, *in vitro*, cytotoxicity effects against *P. oryzae*. The isolate *P. viridicatum* CSE_{74} 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}.

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

Endophyte species	Isolate (s)	Host plant species	Plant segment	Location (Iran)
P. aurantiogriseum	CAE_{39}	*C. arizonica*	Twig	Fars
P. chrysogenum	CAE_{85}	*C. arizonica*	Stem	Hamedan
P. commune	CAE_{13}	*C. arizonica*	Stem	Hamedan
	CSE_{88}	*C. s.* var. cereiformis	Stem	Fars
	CAE_{12}	*C. arizonica*	Stem	Markazi
P. echinulatum	CAE_{17}	*C. arizonica*	Stem	Hamedan
	POE_{28}	*T. orientalis*	Leaf	Hamedan
	CSE_{97}	*C. s.* var. cereiformis	Twig	Fars
P. expansum	POE_{34}	*T. orientalis*	Twig	Fars
	CAE_{1}	*C. arizonica*	Leaf	Hamedan
P. viridicatum	CSE_{65}	*C. s.* var. cereiformis	Twig	Fars
	CSE_{74}	*C. s.* var. fastigiata	Twig	Fars
	CSE_{71}	*C. s.* var. cereiformis	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 (µg ml^{-1})					
		250.0	125.0	62.5	31.2	15.6	7.8
CSE_{74}	*P. viridicatum*	*	*	*	+++	++	-
CSE_{71}	*P. viridicatum*	*	*	+++	++	++	+
CSE_{88}	*P. commune*	*	*	+++	++	+	+
CAE_{12}	*P. commune*	*	+++	+++	++	+	-
CAE_{1}	*P. expansum*	*	+++	+++	++	+	-
POE_{34}	*P. expansum*	*	+++	++	++	-	-
CAE_{85}	*P. chrysogenum*	*	+++	++	++	-	-
CAE_{39}	*P. aurantiogriseum*	+++	+++	++	+	-	-
CAE_{17}	*P. echinulatum*	+++	+++	++	-	-	-
POE_{28}	*P. echinulatum*	+++	++	++	-	-	-
CAE_{13}	*P. commune*	+++	++	-	-	-	-
CSE_{97}	*P. echinulatum*	++	++	-	-	-	-
CSE_{65}	*P. viridicatum*	+	+	-	-	-	-

Symbols: (*) The *P. oryzae* conidial germination was inhibited; (+++) strong growth inhibition of germ tube (≤ 1/3 of control); (++) moderate growth inhibition of germ tube (1/3-2/3 of control); (+) low growth inhibition of germ tube (≥ 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 CSE_{74} and CSE_{71}), *P. aurantiogriseum* (isolate CAE_{39}), *P. commune* (isolates CSE_{88} and CAE_{12}) and *P. expansum* (isolates CAE_{34} and POE_{1}) showed, *in vitro*, cytotoxicity effects against *P. oryzae*

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(Table 4). The isolate *P. viridicatum* CSE_{74} showed the most significant bioactivity among all Penicillia. Intracellular metabolites of *P. viridicatum* CSE_{74} 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* CSE_{71} was stronger than that of its extracellular metabolite and comparable to that of extracellular metabolite of *P. viridicatum* CSE_{74}. Remarkably, *P. aurantiogriseum* CAE_{39}, which didn't show cytoxicity by its extracellular metabolite at high concentration of 250.0 µg/ml, exhibited cytoxicity by its intracellular metabolite at this range. However, the intracellular metabolites of both *P. commune* CSE_{88} and *P. chrysogenum* CAE_{85} showed less cytotoxic effects compared to their extracellular metabolites. Surprisingly, *P. viridicatum* CSE_{65}, which was isolated from the same host species as isolate CSE_{71}, 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* CSE_{74} was superior to others (Table 5). *P. viridicatum* CSE_{71}, *P. viridicatum* CSE_{65}, *P. aurantiogriseum* CAE_{39} and *P. commune* CAE_{12} 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* CSE_{74}, *P. viridicatum* CSE_{71} and surprisingly *P. commune* CAE_{13} had the highest effects on *D. seriata*. The endophytic *P. viridicatum* CSE_{71} was the second isolate showing a significant antifungal effect on *S. viticola*. Meanwhile, *P. viridicatum* CSE_{71}, *P. echinulatum* CSE_{17} and *P. chrysogenum* CAE_8 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}	*P. viridicatum*	*	*	*	*	+++	++
CSE_{71}	*P. viridicatum*	*	*	*	+++	+++	++
CAE_{39}	*P. aurantiogriseum*	*	+++	+++	++	-	-
CSE_{88}	*P. commune*	*	+++	+++	+	-	-
CAE_{12}	*P. commune*	*	+++	++	+	-	-
POE_{34}	*P. expansum*	*	+++	+	+	-	-
CAE_1	*P. expansum*	*	++	+	-	-	-
CAE_{13}	*P. commune*	+++	+++	++	+	-	-
CAE_{85}	*P. chrysogenum*	+++	+++	++	-	-	-
CAE_{17}	*P. echinulatum*	+++	++	+	-	-	-
CSE_{97}	*P. echinulatum*	++	++	+	-	-	-
POE_{28}	*P. echinulatum*	++	+	-	-	-	-
CSE_{65}	*P. viridicatum*	+	+	+	-	-	-

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

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

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

Data (significant at $P \leq 0.01$) are averages (± 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 Penicillia	Cypress phytopathogenic fungi Radius of mycelial growth (mm) and growth inhibition (%)		
		S. viticola	***P. cupressi***	***D. seriata***
CSE_{74}	*P. viridicatum*	12.3 ± 0.6 (71)^{a}	14.3 ± 1.1 (67)^{a}	15.3 ± 1.1 (65)^{a}
CSE_{71}	*P. viridicatum*	13.6 ± 1.1 (68)^{ab}	15.6 ± 0.6 (64)^{ab}	16.0 ± 0.0 (63)^{a}
CAE_{17}	*P. echinulatum*	14 ± 1.0 (68)^{b}	15.7 ± 0.6 (64)^{ab}	19.3 ± 1.1 (56)^{bc}
CSE_{65}	*P. viridicatum*	14.3 ± 1.5 (67)^{bc}	16.3 ± 0.6 (62)^{bc}	19.6 ± 1.5 (55)^{bc}
CAE_{85}	*P. chrysogenum*	14.6 ± 0.6 (66)^{bc}	15.6 ± 1.0 (64)^{ab}	20.3 ± 0.6 (53)^{bcd}
CSE_{97}	*P. echinulatum*	15.6 ± 0.6 (64)^{cd}	16.3 ± 0.6 (62)^{bc}	20.3 ± 0.6 (53)^{bcd}
CAE_{39}	*P. aurantiogriseum*	15.6 ± 1.1 (64)^{cd}	17.3 ± 1.5 (60)^{bc}	20.6 ± 0.6 (53)^{cd}
CSE_{88}	*P. commune*	15.6 ± 0.6 (64)^{cd}	17.7 ± 1.0 (59)^{cd}	21.3 ± 0.6 (51)^{d}
CAE_{13}	*P. commune*	15.6 ± 0.6 (64)^{cd}	19.3 ± 1.1 (55)^{de}	16.6 ± 1.1 (62)^{a}
CAE_{12}	*P. commune*	17 ± 0.0 (61)^{de}	17.3 ± 1.1 (60)^{bc}	19.3 ± 0.6 (56)^{bc}
POE_{28}	*P. echinulatum*	17 ± 1.0 (61)^{de}	19.3 ± 1.1 (55)^{de}	22.3 ± 0.6 (49)^{e}
CAE_{1}	*P. expansum*	17.3 ± 0.6 (60)^{e}	17.6 ± 2.1 (59)^{cd}	19.0 ± 1.7 (56)^{b}
POE_{34}	*P. expansum*	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 (± standard deviation) of three replicates. Similar letters indicate no significant difference. Figures in parentheses are pathogen growth inhibition (%).

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Antimicrobial activities of intra- and extra-cellular metabolites of the endophytic Penicillia

Metabolites extracted from the superior Penicillia isolates (*P. viridicatum* CSE_{74} and *P. viridicatum* CSE_{71}) 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* CSE_{74} 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 (µg mL^{-1})			
		MIC[a]		MBC[b]	
		Extracellular	Intracellular	Extracellular	Intracellular
P. viridicatum CSE_{74}	*Pseudomonas syringae*	15.6	31.2	62.5	62.5
	Erwinia amylovora	15.6	31.2	31.2	62.5
	Bacillus sp.	31.2	62.5	62.5	125
P. viridicatum CSE_{71}	*Pseudomonas syringae*	15.6	31.2	62.5	62.5
	Erwinia amylovora	31.2	62.5	62.5	125
	Bacillus 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 IC_{80} 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 Cuppresaceae 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 *Penicillum citreoni* and *P. freii* have been isolated, as endophytic fungi, from the cypress *Platycladus oreintalis* (Syn. *T. orientalis*) (Hoffman & Arnold, 2008;

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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 *Penicillum 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* CSE_{74} and CSE_{71} 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* CSE_{74} was significantly superior to the other species. The fungi *P. viridicatum* CSE_{74}, *P. viridicatum* CSE_{71} and *P. commune* CAE_{13} showed the highest antagonistic effects on *D. seriata*. The fungi *P. viridicatum* CSE_{71}, *P. echinulatum* CSE_{17} and *P.chrysogenum* CAE_{8} showed similar significant effects on *P. cupressi*. Interestingly, *P. viridicatum* CSE_{65} 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* CSE_{74} and *P. viridicatum* CSE_{74}, 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.

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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 جوانه‌زنی 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 *Spencermartinsia 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 ضدجوانه‌زنی کنیدیوم، بیوکنترل

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