Scientific Papers. Series B, Horticulture. Vol. LX, 2016
Print ISSN 2285-5653, CD-ROM ISSN 2285-5661, Online ISSN 2286-1580, ISSN-L 2285-5653

ACTIVE FUNGAL ENDOPHYTES AGAINST PHYTOPATHOGENIC FUNGI- DWELLERS OF ROMANIAN AND CANARIAN *ARTEMISIA* SPP.

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Abstract

Endophytic fungi have been isolated from Artemisia austriaca (isolates HRO184, HRO183, HRO169 and HRO115), A. vulgaris (isolate HRO158) and A. thuscula (isolates HLP7, HLP26, HLP27, and HLP44) in Romania and La Palma, Canary Islands, Spain. The strains were studied for their bioactivity against phytopathgenic fungi (Alternaria alternata, Alternaria dauci, Alternaria brassicicola, Fusarium oxysporum, Fusarium solani, Fusarium moniliforme, Sclerotinia sclerotiorum, Botrytis cinerea, Phoma sp., Geotrichum sp. and Cladosporium sp.) using dual culture, agar dilution and volatile compounds (VOC) techniques. In dual culture assays, all Canarian isolates were strong antagonists of at least one pathogen and two Romanian isolates, HRO169 against S. sclerotiorum and HRO158 against A. brassicicola. In VOC assays, a difference was observed between slow growth isolates (1-2 cm/week) and normal growth (4-5 cm/week), therefore three moments of fungal pathogen inoculation were used: after three and five days from the endophyte inoculation. The highest inhibition gradually produced by the volatile compounds was observed with HLP44 isolate against Cladosporium sp. (%I = 31.5) at 7th day. Solvent extracts were obtained from HRO169, HRO158, HLP44, HLP27 and HLP7 isolates and further two of them (HRO158 and HLP44) were fractionated using vacuum-liquid chromatography eluted with n-hexane:EtAc:MeOH gradient to give seven fractions. Extract obtained from HLP44 isolate strongly inhibited A. brassicicola (%I = 44), F. moniliforme (%I =52.78) and S. sclerotiorum (%I = 50.4), at 0.1mg ml^{-1}. Ethyl acetate fraction was the most active against A. brassicicola, F. solani and B. cinerea (%I = 42.68, 59.17 and 49.36, respectively, at 0.1mg ml^{-1}) followed by the EtAc:MeOH (90:10) fraction which also inhibited A. brassicicola and F. solani (%I = 35.83 and 39.53, respectively).

Key words*: Artemisia*, *bioactivity*, *endophytic fungi*, *phytopathogens*

INTRODUCTION

Fungal endophytes are microorganisms that live in the intercellular spaces of stems, petioles, roots and leaves of plants without causing observable manifestation of their existence (Strobel and Long, 1998). Same substances can be produced by both endophytes and plants (Stierle et al, 1993; Strobel et al, 1997; Lee et al, 1995; Kusari et al, 2008). According to Strobel (2002), plants with medicinal value or unusual longevity, plants that survive under extreme conditions often harbour potential fungal endophytes that produce bioactive metabolites. Medicinal plants are revealed as host endophytes which in turn provide protection from infectious agents (Strobel et al, 2002). A cavalcade of endophytic species dwelling in medicinal plants revealed bioactivity features in various studies (Li et al, 2005; Raviraja et al, 2006; Chowdhary and Kaushika, 2015; Purwantini et al, 2015). The symbiotic relation between the endophyte and its host is considered 'defensive mutualism' id est the resistance of the host to pathogens, phytophagous insects and environmental conditions increases; secondary metabolites are involved in most cases (Gonzalez-Coloma et al, 2016). An array of compounds belonging to various chemical groups are metabolized by endophytic fungi such as phenols, steroids, flavonoids, quinones, terpenoids, xanthones, peptides, alkaloids, aliphatic compounds, phenylpropanoids, isocoumarins, benzopyranones, tetralones, cytochalasines and

enniatines (Schulz et al. 2002, Rocha et al. 2011, Schulz and Boyle 2005, Aly et al. 2010, Santos et al, 2003). New compounds were isolated from endophytes inhabiting *Artemisia annua* (Ge et al. 2010; Lu et al, 2000) and *Erythrina crista-galii* (Weber et al, 2004). The production of antioxidant compounds by plants, like phenolic acids and their derivatives (Huang et al., 2007), isobenzofuranones (Strobel et al., 2002), isobenzofurans (Harper et al., 2003), as well as mannitol and other carbohydrates (Richardson et al., 1992), is attributed to the presence of reactive oxygen species (ROS) generated by endophytes (Gonzalez-Coloma et al, 2016). Volatile compounds (VOCs) are also produced by fungal endophytes among other microorganisms, but less is known about the pathways in which they are produced. Many of them are either metabolic transformation products of lipids, proteins, heterocyclic metabolites or other components of living tissues or degradation end-products ('waste products') of fungal catabolic pathways (Bennett et al, 2012). Species of endophytes produce antifungal and antimicrobial VOCs like *Muscodor albus* (Strobel et al, 2001), *Oxyporus latemarginatus* (Lee et al, 2009) and *Gliocladium* sp. (Stinson et al, 2003), respectively. *Artemisia* is a wide studied genus of plants for its medicinal and bioactive properties (Soylu et al, 2005; Brudea, 2008; Dancewicz and Gabrys, 2008; Garcia et al, 2015; Abad et al, 2012) and recently its endophytic fungi communities have been taken into observation looking for bioactivity features (Haniya et al, 2013; Qian et al, 2014; Purwantini et al, 2015, Cosoveanu et al, 2016). The present study selected several species of fungal endophytes previously isolated from *Artemisia austriaca*, *Artemisia vulgaris* and *Artemisia thuscula* to evaluate their bioactive potential. It is noteworthy to mention that is the first notification on fungal endophytes isolated from *A. austriaca* and *A. thuscula* and their bioactivity.

MATERIALS AND METHODS

Plant sampling and isolation techniques

Plant samples were collected and processed in 2012 in Romania: *A. austriaca* from Tuzla, Murighiol, Babadag Lake; *A. vulgaris* from Mahmudia; and in La Palma Island (Spain): *A. thuscula* from La Galga and Tigalate. A surface sterilization method was used in order to suppress epiphytic microorganisms from the plant samples. Briefly, plants were immersed first in sterile H2O, followed by 1min in EtOH 70%, 1min in sodium hypochlorite 15%, 1 min in EtOH at 70% and finally washed with sterile H2O (changed from Schulz et al., 1993). The isolation procedure was performed according to Cosoveanu et al. (2014). In order to analyse the fungal diversity, each replicate obtained from distinct stem fragments was registered. When an endophyte was acquired in pure culture it was preserved (Czapek, T=5°C and Glycerol 20% DI H_2O, T= -30°C), bioactively tested and identified.

Dual culture assays

Dual culture technique was employed to find endophytic fungi that produce metabolites which inhibit *S. sclerotiorum*, *F. oxysporum*, *F. moniliforme*, *F. solani*, *A. brassicicola*, *A. dauci*, *A. alternata*, *Phoma* sp. *Geotrichum* sp. and *Cladosporium* sp. mycelial growth *in vitro*. PDA plates were incubated at 25ºC in darkness for 7 days and observed daily; plates were left for a further week to check the stability of the interaction. The following criteria based on Kusari et al. (2013) were used to interpret the results:

0-Mycelia grow until making contact with each other
1-Mutual inhibition (both mycelia stop growing at a certain distance)
2-Mycelia grow until making contact with each other and in the area where the contact is produced morphological changes occur/ the growth is stopped in a convex form
3-Pathogen growth is detained at a certain distance from the endophyte (<2 mm)
4-Pathogen growth is detained at a certain distance from the endophyte (>2 mm)
RDP- Rapid development and parasitism of the endophyte
RD- Rapid development of the endophyte
RDL- Rapid development of the endophyte and lysed mycelia of the pathogen
L- Opponent fungus presents lysed mycelia
P- Endophyte displays parasitism on pathogen

It should be noted that also the pathogen may respond similarly to criteria 3, 4, RDP, RD, RDL, L and P (further, the results for this case are

noted with *). However, this study focuses only on the endophyte response towards pathogen.

Fungal isolates

Fungal isolates (endophytic and pathogenic strains) were maintained on PDA, T=25°C, in darkness. Endophytes were selected based on their results in preliminary assays of antagonism. Pathogens were chosen due to their different interactions with the host and their high economic importance: *Alternaria alternata*, *A. dauci*, *A. brassicicola*, *Fusarium oxysporum*, *F. moniliforme*, *F. solani*, *Sclerotinia sclerotiorum, Botrytis cinerea, Phoma* sp, *Geotrichum* sp. and *Cladosporium* sp.

Biometric agar dilution assays

Tests were carried out to determine the biological activity of extracts using biometric agar dilution method. The extracts were incorporated into PDA as follows: 1, 0.5 and 0.1 mg ml^{-1}. The final percentage of ethanol in the media was adjusted to a concentration of 1% (v/v). Plates containing the solvent (ethanol) were used as negative control. Each pathogen was spot - inoculated at eight equidistant points to PDA media amended with the fungal extracts at tested concentrations. Three replicates were used per treatment. For each extract and concentration, inhibition of radial growth (%I) compared with the control was calculated after 72 hours of incubation at 25ºC, in the dark. The radial growth was measured with an image - processing software Image J -Wayne Rasband (NIH).

Kruskal-Wallis Test and Mann Whitney U were performed using IBM SPSS Statistics 21.0.

Volatile compounds assays (VOC)

The VOC assays analyse the activity of volatile compounds produced by the endophytes on phytopathogens. The assays were done in Petri plates, using only the plate bases with nutritive media for the fungal inoculation as following: plate base with endophyte + plate base with pathogen and the controls: base without pathogen + base with endophyte and correspondently for the pathogen. Plates were incubated for a week at T=25°C, in darkness. Assays were performed in triplicates. Measurements were made daily, calculating an average of two measurements of the same inoculum diameter. In the case of slow growth endophytic fungi three different moments for the pathogen inoculation were chosen allowing to the endophytic fungus to develop more as follows: inoculation of both fungi at the same time (Moment 0), inoculation of the pathogen after three days from the inoculation of the endophyte (Moment 1) and inoculation of the pathogen after five days from the endophyte's inoculation (Moment 2).

The following criteria were used to interpret the results:

A = E<CE: The pathogen inhibits the endophyte
B = P<CP: The endophyte inhibits the pathogen
C = CE<E: The pathogen enhances the endophyte's growth
D = CP<P: The endophyte enhances the pathogen's growth
* CE: Control of endophyte; CP: Control of pathogen; E: Endophyte in interaction; P: Pathogen in interaction

High scale cultivation of endophytic fungal isolates

Five of nine fungal isolates showing antagonistic activity were further explored for bioactivity of their crude extracts by multiplication on rice media. Rice medium was prepared in 500ml Erlenmeyer flasks containing 120g of rice grains with 40ml H_2O (autoclaved). The flasks were inoculated with 4-6 disks of endophytic fungus mycelium (25°C, darkness for three weeks).

Chemical solvent extraction of endophytic fungal isolates (crude extract and fractionation)

120ml of ethyl acetate (Sigma Aldrich) was poured on the rice grains covered with the fungus mycelium and kept aside for 24h. The content was filtered under vacuum using a Buchner funnel and the solvent extraction was repeated thrice. The collective extract was dried up with vacuum rotary evaporator under reduced pressure at 50°C and used as crude extract for further evaluation. Two of the crude extracts were selected to be fractionated. The extracts were previously subjected to partitioning between n-hexane and MeOH (V/V) to remove the fatty acids, twice. The MeOH fraction was chromatographed on a SiO_2 vacuum-liquid chromatography column (VLC) eluted with n-

hexane:EtAC:MeOH gradient to give seven fractions (three times the volume of the column per solvent fraction). Fr-1 (n-hex. 100%), Fr-2 (n-hex:EtAc 90:10), Fr-3 (n-hex:EtAc 75:25), Fr-4 (n-hex:EtAc 50:50), Fr-5 (EtAc 100%), Fr-6 (EtAc:MeOH 90:10), Fr-7 (MeOH 100%). Fractions were further treated as the crude extracts.

RESULTS AND DISCUSSIONS

In dual culture assays, isolate HLP44 inhibited the development of six pathogens with a higher distance than 2mm (criterion 4) and three with a distance of 2mm (criterion 3) out of 11 pathogens (Table 4). Isolates HLP7 and HLP26 were also strong inhibitors of four and three pathogens, respectively. Also HLP27 and HRO169 isolates impeded the development of one pathogen each. In the agar dilution assays, HRO169 (extract code-1090) inhibited (%I= 47.70, at 0.1mg/ml) only *F. oxysporum* although the results from dual culture did not predict it (criterion 1). The crude extract of HLP7 (code- 1114) inhibited *A. brassicicola* (%I= 32.55, at 0.1mg/ml) but not *Cladosporium* sp. (%I= 9.7, at 1mg/ml) although in dual culture the observed reactions were similar for both pathogens (criterion 4). Despite the interesting activity in dual culture (criterion 3), the extract was not active against *A. alternata* and *A. dauci*. (%I= 15.5 and 5.02, respectively; at 1mg/ml). HLP44 extract (code- 1092) was the most active and therefore it was fractionated. 1092 inhibited with interesting values seven of the nine tested pathogens (Table 3). Although all three species of *Alternaria* reacted in a similar manner in dual culture assay, the response of the extract in dilution agar assays variated (vs. *A. alternata* %I= 38.6, vs. *A. brassicicola* %I= 41.7. vs. *A. dauci* %I= 19.5, at 1mg/ml). As for the three species of *Fusarium*, results of inhibition were similar (Table 3). The bioactivity of this extract was disjoined, having various active fractions with different pathogens (Table 3). Briefly, the fraction which most inhibited the mycelial growth of tested pathogens with interesting values was the one eluted with ethyl acetate (vs. *A. brassicicola* %I= 42.7, vs. *F. solani* %I= 59.2, vs. *F. moniliforme* %I= 43.2 and vs. *B. cinerea* %I= 49.4, at 0.1mg/ml). Ethyl acetate and methanol extracts of an endophytic *Chaetomium globosum* isolate were more effective than hexane extract against *S. sclerotiorum* (Kumar et al, 2013). Yet, the non-polar fractions (L0 and F2, eluted with hexane and n:hexane-EtAc 90:10, respectively) strongly inhibited *B. cinerea* (L0- %I= 35.1; F2- %I= 43.4, at 0.1mg/ml). Previous reports on hexane extracts obtained from *Colletotrichum globosum* showed antifungal properties against *B. cinerea* (Nakashina et al, 1991). Fungal endophytes were tested for their VOCs bioactivity and separated in two groups due to their type of growth: regular growth and slow growth. It was hypothesized that the more mass of endophytic mycelia is produced, the more VOCs would be generated a posteriori in the interaction with the pathogen. In the slow growth group the endophytes were left to develop in the absence of the pathogen for three and five and five days. The highest percentage of inhibition, reached gradually, (%I= 31.5, at Moment 3) was calculated for the interaction between *Cladosporium* sp. and HLP44, confirmed by Kruskal Wallis test ($p= 0.027$). Overall there were no significant differences between the inoculation moments, but neither interaction with high percentages of inhibition. In the group of regular growth, the interactions between the same pathogen and two endophytes were compared. No significant difference resulted ($p<0.05$), therefore no difference in the sensibility of the pathogen exposed to more than one reputed bioactive endophytes, was found. The structural groups of VOCs detected in different individuals of *A. vulgaris* collected from various countries and regions show similarities as the main groups belong to monoterpenes followed by sesquiterpenes (Zhigzhitzhapovae et al, 2016). *Artemisia* spp. essential oils have antimicrobial properties (Baykan Erel et al, 2010), antiparasitic and cytotoxic activity (Martinez-Diaz et al, 2015) so the community of harboured endophytes seems likely to be also producing bioactive VOCs, as previously shown (Strobel et al, 2011).

Table 1. VOC interaction between pathogens and endophytes with regular growth: percentages of inhibition (expressed as average and Standard deviation STD) and criteria of interpretation

	%I pathogens- AVG (STD)			%I endophytes- AVG (STD)			CRITE-RIA
Interaction	Day 1	Day 4	Day 7	Day 1	Day 4	Day7	
A. alternata &HLP26	20.74 (3.03)	2.86 (6.92)	2.33 (5.58)	-3.33 (4.71)	10.56 (2.66)	17.53 (3.91)	
A. alternata & HRO158	19.16 (4.43)	9.65 (4.50)	9.32 (3.05)	40.99 (14.62)	44.18 (39.66)	6.07 44.92)	
A. brassicicola & HRO158	7.69 (10.88)	-8.11 (8.63)	19.21 (5.33)	6.78 (12.04)	0.86 (5.17)	-3.15 (6.07)	B
A. brassicicola & HLP26	-18.59 (4.53)	-18.62 (19.19)	2.61 (11.18)	0.00 (0.00)	21.07 (10.44)	16.96(4.67)	
A. dauci & HRO169	3.42 (2.42)	5.12 (5.13)	0.49 (6.85)	3.04 (2.15)	-7.91 (7.22)	-15.64 (6.22)	
A. dauci & HLP26	-2.41 (15.29)	-1.50 (8.77)	-2.07 (6.39)	0.00 (0.00)	5.25 (3.84)	11.36 (2.09)	
F. oxysporum & HLP26	-30.37 (35.09)	1.53 (1.24)	17.16 (3.74)	3.33 (4.71)	28.82 (3.09)	44.33 (4.99)	A
F. oxysporum & HRO184	-10.74 (34.91)	10.27 (1.98)	9.07 (5.64)	-0.67 (8.26)	9.60 (5.00)	27.69 (3.17)	A
F. moniliforme & HRO158	-4.22 (9.71)	-0.11 (3.04)	2.55 (8.48)	-7.77 (14.87)	-5.47 (18.68)	-18.49 (19.38)	
F. solani & HLP26	-3.92 (7.02)	0.02 (3.74)	5.94 (1.38)	-20.00 (0.00)	9.06 (1.69)	22.19 (4.93)	A
F. solani & HRO184	-1.96 (5.11)	-2.06 (4.03)	7.15 (2.90)	-8.08 (18.24)	-7.23 (8.23)	8.31(7.22)	
B. cinerea & HRO158	27.23 (15.87)	-16.94 (14.07)	-21.89 (14.24)	6.66 (24.97)	-11.05 (30.42)	-15.70 (17.65)	
B. cinerea & HRO183	10.65 (11.44)	-7.38(10.86)	-10.03 (12.71)	-2.04 (6.73)	10.04(1.41)	7.47 (4.82)	
S. sclerotiorum & HRO158	-39.91 (60.20)	-20.20(28.57)	-20.20 (28.57)	4.40 (14.92)	10.42(26.87)	17.22 (58.66)	
S. sclerotiorum & HRO169	-33.07 (65.05)	-18.18 (25.71)	-18.18 (25.71)	6.73 (11.59)	6.29 (1.45)	2.36 (3.61)	

Table 2. VOC interaction between pathogens and endophytes with slow growth: percentages of inhibition (expressed as average %I and standard deviation STD), moments of inoculation of pathogen and criteria of interpretation.

		%I pathogen			%I endophyte			
Interaction	Moment	Day 1	Day 4	Day 7	Day 1	Day 4	Day7	CRITERIA
A. brassicicola	M0	15.0 (13.2)	17.1 (4.8)	6.8 (15.3)	0.0 (0.0)	15.4 (8.2)	-4.4 (16.1)	
& HLP44	M1	-0.8 (8.9)	36.2 (1.5)	33.2 (4.4)	13.5 (8.2)	2.7 (9.9)	-0.3 (9.5)	B
	M2	-3.0 (5.2)	35.3 (11.8)	24.1 (4.1)	2.4 (14.8)	-0.3 (9.5)	-1.9 (12.9)	B
A. brassicicola	M0	20.1 (1.3)	23.0 (7.6)	-1.2 (5.9)	0.0 (0.0)	4.5 (5.1)	5.8 (3.3) a	
& HLP27	M1	-2.3 (26.6)	24.3 (9.5)	8.7 (24.8)	-1.2 (4.0)	3.9 (3.8)	15.2 (3.1)b	
	M2	-18.2 (18.2)	16.7 (3.7)	25.2 (1.5)	-8.6 (8.1)	-9.3 (5.4)	-6.6 (8.3)c	B
A. dauci	M0	23.3 (2.9)	15.9 (9.6)	2.6 (20.9)	0.0 (0.0)	5.7 (5.6)	-0.7 (9.0)	
& HLP44	M1	16.7 (3.6)	19.4 (3.6)	5.6 (6.3)	9.6 (8.9)	12.8 (4.8)	12.4 (5.0)	
A. dauci	M0	6.7 (11.5)	1.5 (10.3)	6.5 (4.7)	0.0 (0.0)	-92.5 (62.3)	-77.8 (79.1)	
& HLP7	M1	20.8 (7.2)	-4.7 (6.4)	-3.0 (3.9)	-92.3 (60.1)	-73.0 (68.4)	-68.7 (72.7)	
	M2	9.1 (0.0)	8.4 (1.8)	9.8 (4.2)	-104.5 (83.5)	-119.1 (94.2)	-131.1 (109.0)	
B. cinerea	M0	18.5 (29.4)	2.5 (5.4)	2.5 (5.4)	-8.3 (14.4)	-4.7 (4.1)	-5.2 (1.2)	
& HLP27	M1	2.2 (3.8)	6.5 (12.3)	4.4 (12.7)	-1.2 (4.0)	-2.0 (5.3)	13.5 (4.7)	
	M2	8.9 (10.2)	3.6 (6.4)	1.8 (6.4)	-3.3 (5.0)	-8.7 (6.2)	-11.2 (10.2)	
Cladosporium sp.	M0	16.2 (16.7)	8.3 (15.7)	3.0 (10.5)a	0.0 (0.0)	4.2 (7.2)	-5.6 (20.6)	
& HLP44	M1	2.5 (13.1)	17.7 (7.7)	17.1 (4.8)b	1.5 (12.4)	3.0 (5.2)	4.7 (4.6)	
	M2	-18.6 (25.2)	15.1 (2.0)	31.5 (3.2)c	10.3 (13.7)	13.8 (7.6)	15.0 (6.2)	B
Cladosporium sp.	M0	-11.9 (33.0)	0.0 (23.8)	-5.5 (13.1)	0.0 (0.0)	-18.1 (28.5)	16.2 (22.3)	
& HLP7	M1	14.1 (4.4)	11.0 (1.7)	6.6 (12.2)	-88.3 (80.6)	-103.5 (116.9)	-122.8 (137.8)	
	M2	-12.8 (23.4)	2.0 (10.1)	16.2 (21.4)	-30.2 (62.1)	-46.6 (85.5)	-45.0 (86.2)	

Values with different letter have statistical difference P<0,05, U Mann Whitney Test

Table 3. Dilution agar assays with crude extract and fractions versus fungal pathogens- %I (STD)

Extract	[C]	A. a.	A. b.	A. d.	F. o.	F. s.	F. m.	S. s.	B. c.	Clad.
972	1 mg/ml	20.8 (5.8)						12.2(9.4)		
1090	1 mg/ml	29.8(4.1)	12.8(5.2)		45.8(3.3)a	23.3(7.8)a	16.2(7.4)	14.8(5.8)		
	0.5 mg/ml				57.0(5.2)b	31.2(6.0)b				
	0.1 mg/ml				47.8(4.8)a	6.3(7.8)c				
1091	1 mg/ml		44.5(4.7)	-6.6(8.9)				2.6(9.9)	1.4(8.6)	
1114	1 mg/ml	15.5(2.6)a	42.3(8.0)a	5.0(4.6a						9.7(12.5)a
	0.5 mg/ml	18.7(2.8)a	41.2(5.7) b	11.7(4.6) b						13.1(17.1)a
	0.1 mg/ml	18.0(2.8)a	32.6(4.3) b	4.8(4.6)a						-16.4(29.5) b
1092	1 mg/ml	38.6(1.6)a	41.7(6.1)a	19.5(4.1)a	37.9(10.2)a	34.3(5.5)a	42.5(7.2)a	71.5(8.0)a	45.2(2.5)a	
	0.5 mg/ml	29.6(1.2) b	46.0(5.3)a	13.8(*3.0)b	26.6(6.5) b	18.4(4.2)b	28.7(6.9)b	67.9(8.8)a	51.1(5.0)b	13.4(10.9)a
	0.1 mg/ml	17.2(2.0)c	44.0(5.3)a	3.5(*2.1)c	16.9(7.5) c	-1.5(4.3) c	52.8(7.8)c	50.4(33.7)a	18.6(8.8)c	25.5(19.0a
1092LO	0.1 mg/ml	-1.0(11.6)	9.2(7.5) *			5.4(6.7)	6.8(7.7)		35.1(11.9) *	
1092F2	0.1 mg/ml	4.8(6.7)	29.3(4.9) *			15.3(5.1) *	2.7(10.2		43.4(5.4) *	
1092F3	0.1 mg/ml	-4.6(4.9)	7.6(4.8) *			7.5(9.2)	0.5(6.8)		14.4(7.1) *	
1092F4	0.1 mg/ml	4.5(5.2)	27.2(4.8) *			34.0(4.5) *	21.2(9.1) *		19.6(2.8) *	
1092F5	0.1 mg/ml	21.5(2.8) *	42.7(3.8) *			59.2(0.9) *	43.2(5.5) *		49.4(3.1) *	
1092F6	0.1 mg/ml	3.2(9.2)	35.8(5.2) *			39.5(5.4) *	10.8(10.7)		32.9(1.6) *	
1092F7	0.1 mg/ml	2.0(5.4)	19.3(3.6) *			24.7(5.3) *	-17.0(6.6)		19.1(5.7) *	

U Mann Whitney was applied in the case of the assays with fractions and pathogens to check the statistical difference between control and treatment (values marked with * have p<0.05) and between treatment at different concentrations (values with different letter have statistical diference p<0.05). A.a.= *A. alternata*, A.b.= *A. brassicicola*, A.d.= *A. dauci*, F.m. = *F. moniliforme*, F.s.= *F. solani*, F.o.= *F. oxysporum*, S.s.= *S.sclerotiorum*, B.c.= *B. cinerea*, Clad= *Cladosporium* sp.

Table 4. Dual culture assay: fungal endophytes and pathogens (interaction criteria)

	HLP7	HLP26	HLP27	HLP44	HRO115	HRO158	HRO169	HRO183	HRO184
F. o.	0	3, P*	2*	3, P*	0	2	1	0	4
F. m.	0	0, P*	2*	3, P*	2	0	0	0	P*
F. s.	0	3, P*	2*	3, P*	0	2	1	0	4
B. c.	4	4	4	4	0	0	2	3, P	2*
S. s.	4	2	2	4	2	2	4	RD, 1	RD*P*
A. a.	3	3	0	4	0	0	3	1	0
A. d.	3	4	P	4	P	3	1	P	2
A. b.	4	4	3	4	P	3, P	3*	P	0
Phoma	4*	4*	4*	1	RD, L	4*	4*	4*, RD	4*
Geot.	4*	4*	4*	1	4*	4*	4*	4*, RD	nt
Clad.	4	1	1	4	P	0	1	P, RD	4*

A.a.=*A. alternata*, A.b.= *A. brassicicola*, A.d.= *A. dauci*, F.m. = *F. moniliforme*, F.s.= *F. solani*, F.o.= *F. oxysporum*, S.s.= *S.sclerotiorum*, B.c.= *B. cinerea*, Clad= *Cladosporium* sp. , Geot= *Geotrichum* sp. 0-Mycelia grow until making contact with each other; 1- Mutual inhibition (both mycelia stop growing at a certain distance); 2- Mycelia grow until making contact with each other and in the area where the contact is produced morphological changes occur/ the growth is stopped in a convex form; 3- Pathogen growth is detained at a certain distance from the endophyte (<2 mm); 4- Pathogen growth is detained at a certain distance from the endophyte (>2 mm); RD- Rapid development of the endophyte; L- Opponent fungus presents lysed mycelia; P- Endophyte displays parasitism on pathogen. *= the pathogen is evaluated with the correspondent criteria; the rest of the cases are applied to the action of the endophyte on the pathogen.

CONCLUSIONS

Our study has shown the potential of two fungal endophytes against important plant pathogens. The most interesting fungal endophyte is HLP44 isolate (from *A. thuscula*), as its valences are multiple being a spring of active compounds against *A. brassicicola*, *F. solani* and *B. cinerea* found mainly in the ethyl acetate fraction but also in the hexane fraction. More, this isolate strongly inhibited *B. cinerea*, *S. sclerotiorum*, *A. alternata*, *A. dauci*, *A. brassicicola* and *Cladosporium* sp. in dual

culture and *Cladosporium* sp. in VOC assay which converts it into a tool for biocontrol. Further studies will be carried out to identify the active compounds responsible for the inhibition of the mycelium growth of pathogens. One good candidate for *in vivo* further assays would be HRO158 isolate (from *A. vulgaris*) which inhibited the growth of the mycelium of *A. brassicicola* and *A. dauci* in dual culture assay.

ACKNOWLEDGEMENTS

This research work was carried out partially supported by grant of La Caixa- Fundacion Caja Canarias para Posgraduados (2014).

REFERENCES

Abad M.J., Bedoya L.M., Apaza L., Bermejo P., 2012. The *Artemisia* L. genus: A review of bioactive essential oils. Molecules 17, 2542-2566 doi:10.3390/molecules17032542.

Aly A.H., Debbab A, Kjer J., Proksch P., 2010. Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fungal divers 41, 1–16.

Baykan Erel S., Reznicek G., Senol S.G., Karabay Yavasogulu N.U., Konyalioglu S., Zeybek A.U., 2012. Antimuctobial and antioxidant properties of *Artemisia* L. species from western Anatolia. Turk J Biol 36: 75-84

Bennett J.W., Hung R., Lee S., Padhi S., 2012. Fungal and bacterial volatile organic compounds: an overview and their role as ecological signaling agents. In: Fungal Associations, 2nd Edition. The Mycota IX DOI: 10.1007/978-3-642-30826-0_18.

Brudea V., 2008. The efficacy of some biopesticides and vegetal metabolites in the control of spireas aphid aphis *Spiraephaga* Muller (O. Homoptera – F. Aphididae). Lucrari Stiintifice, vol. 52, seria Agronomie, 611-616.

Chowdhary K., Kaushik N., 2015. Fungal endophyte diversity and bioactivity in the indian medicinal plant *Ocimum sanctum* Linn. PLOS ONE 10(11): e0141444. doi:10.1371/journal.pone.0141444.

Cosoveanu A., Hernandez M., Iacomi-Vasilescu B., Zhang X., Shu S., Wang M., Cabrera R. 2016. Fungi as endophytes in Chinese *Artemisia* spp.: juxtaposed elements of phylogeny, diversity and bioactivity. Mycosphere 7(2): 102–117.

Cosoveanu A., Gimenez-Marino C., Cabrera Y., Hernandez G., Cabrera R., 2014. Endophytic fungi from grapevine cultivars in Canary Islands and their activity against phytopathogenic fungi. International Journal of Agriculture and Crop Sciences. 7(15): 1497-1503.

Dancewicz K., Gabrys B., 2008. Effect of extracts of garlic (*Allium sativum* L.), wormwood (*Artemisia absinthium* L.) and tansy (*Tanaceum vulgare* L.) on the behaviour of the peach potato aphid *Myzus persicae* (Sulz.) during the settling on plants. Pestycydy/Pesticides, (3-4): 93-99

Garcia J.J., Andres M.F., Ibanez A., Gonzalez-Coloma A., 2015. Selective nematocidal effects of essential oils from two cultivated *Artemisia absinthium* populations. Zeitschriftung fur Naturforschung. 70(9) DOI: 10.1515/znc-2015-0109.

Ge H.M., Peng H., Guo Z.K., Cui J.T., Song Y.C., Tan R.X., 2010. Bioactive alkaloids from the plant endophytic fungus *Aspergillus terreus*. Planta Med 76, 822–82.

Gonzalez-Coloma A., Cosoveanu A., Cabrera R., Gimenez C., Kaushik N., 2016. Endophytic Fungi and their Bioprospection. In: Deshmukh S.K., Misra J.K., Tewari J.P., Papp T., (Eds.), Fungi: Applications and Management Strategies. CRC Press, 14-31

Haniya A.M.K., Padma P.R., 2013. Free radical scabenging activity of *Artemisia vulgaris*, L. leaf extract. World Journal of Pharmacy and Pharmaceutical Sciences 2(6): 6381-6390

Harper J.K., Arif A.M., Ford E.J., Strobel G.A., Porco J.A., Tomer D.P., Oneill K.L., Heider E.M., Grant D.M. 2003. Pestacin: a 1,3-dihydro isobenzofuran from *Pestalotiopsis microspora* possessing antioxidant and antimycotic activities. Tetrahedron, 59: 2471–2476.

Huang W.Y., Cai Y.Z., Xing J., Corke H., Sun M. 2007. A potential antioxidant resource: endophytic fungi from medicinal plants. Econ. Bot., 61, 14–30.

Kumar S., Kaushik N., Proksch P., 2013. Identification of antifungal principle in the solvent extract of an endophytic fungus *Chaetomium globosum* from *Withania somnifera*. Springerplus, 2:37.

Kusari S., Lamshoft M., Zuhlke S., Spiteller M., 2008. An endophytic fungus from *Hypericum perforatum* that produces hypericin, J. Nat. Prod. 71: 159-162.

Kusari P., Kusari S., Spiteller M., Kayser O., 2013. Endophytic fungi harbored in *Cannabis sativa* L.: diversity and potenyial as biocontrol agents against host plat-specific phytopathogens. Fungal Diversity 60:137-151

Lee J.C., Lobkovsky E., Pliam N.B., Stroble G.A, Clardy J., 1995. Subglutinol A and B: immunosuppressive compounds from the endophytic fungus *Fusarium cubglutinans*, J. Org. Chem. 60, 7076-7077.

Lee S.O., Kim H.Y., Choi G.J., Lee H.B., Jang K.S., Choi Y.H., Kim J.C., 2009. Mycofumigation with *Oxysporus latemarginatus* EF069 for control of postharvest apple decay and *Rhizoctonia* root rot on moth orchid. J. Appl Microbiol 106: 1213-1219.

Li H., Qing C., Zhang Y., Zhao Z., 2005.Screening for endophytic fungi with antitumour and antifungal activities from Chinese medicinal plants, World J. Microbiol. Biotechnol. 21 (2005) 1515-1519.

Lu H., Zou W.X., Meng J.C., Hu J., Tan R.X., 2000. New bioactive metabolites produced by *Colletotrichum* sp., an endophytic fungus in *Artemisia annua*, Plant Sci. 151, 67-73.

Martinez-Diaz R.A., Ibanez-Escribani A., Burillo J., Heras L., Prado G., Argullo-Ortuno M.T., Julio L.F.,

Gonzalez-Coloma A., 2015. Trypanocidal, trichomonacidal and cytotoxic components of cultivated *Artemisia absinthium* Linnaeus (Asteraceae) essential oil. Mem Inst Oswaldo Cruz, 110(5): 693-699.

Nakashina N., Moromizato Z., Matsuyama N., 1991. The antifungal substance produced by *Chaetomium trilaterale* var. diporum RC-5 isolated from sclerotia of *Sclerotinia sclerotiorum*. Ann Phytopath Soc Japan, 57:657-662.

Purwantini I., Mustofa W., Asmah R., 2015. Isolation of endophytic fungi from *Artemisia annua* l, and indentification of their antimicrobial compounds using bioautography method. International Journal of Pharmacy and Pharmaceutical Sciences. 7(12): 95-99.

Qian Y., Kang J., Geng K., Wang L., Lei B., 2014. Endophytic fungi from *Artemisia argyi* Levl. et Vant. and their Bioactivity. Chiang Mai J. Sci. 41(4): 910-921.

Raviraja N.S., G.L. Maria, Sridhar K.R., 2006. Antimicrobial evaluation of endophytic fungi inhabiting medicinal plants of the western ghats of India, Eng. Life Sci. 6: 515-520.

Richardson, M.D., Chapman, G.W., Hoveland, C.S., Bacon, C.W., 1992. Sugar alcohols in endophyte-infected tall fescue. Crop Sci., 32: 1060–1061.

Rocha A.C.S., Garcia D., Uetanabaro A.P.T., Carneiro R.T.O., Araújo I.S., Mattos C.R.R., Góes-Neto A., 2011. Foliar endophytic fungi from *Hevea brasiliensis* and their antagonism on *Microcyclus ulei*. Fungal Divers 47:75–84.

Rude M.A., Schirmer A., 2009. New microbial fuels: a biotech perspective. Curr Opin Microbiol 12: 274-281.

Santos R.M., Rodrigues G., Fo E., Rocha W.C., Teixeira M.F.S., 2003. Endophytic fungi from *Melia azedarach*, World J. Microbiol. Biotechnol. 19: 767-770.

Schultz B., Wanke S., Draeger S., Aust H.J., 1993. Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. Mycol. Res. 97 (12): 1447-1450.

Schulz B., Boyle C., Draeger S., Aust H.J., Römmert A.K., Krohn K., 2002. Endophytic fungi: a source of novel biologically active secondary metabolites. Mycol Res, 106:996-04.

Schulz, B., Boyle, C., 2005. The endophytic continuum. Mycological Research 109:661–686.

Singh S.K., Strobel G.A., Knighton B., Geary B., Sears J., Ezra D., 2011. An endophytic *Phomopsis* sp. possessing bioactivity and fuel potential with its volatile organic compounds. Microb Ecol 61:729-739.

Soylu E.M., Yigitbas H., Tok F.M., Soylu S., Kurt S., Batsal O., Kaya A.D., 2005. Chemical composition and antifungal activity of the essential oil of *Artemisia annua* L. against foliar and soil-borne fungal pathogens. J. of Plant Diseases and Protection, 112 (3): 229–239.

Stierle A., Strobel G.A., Stierle D., 1993. Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew, Science 260(5105):214-6.

Stinson E., Exra D., Hess W.M., Sears J., Strobel G., 2003. An endophytic *Gliocladium* sp. of *Eucryphia cordifolia* producing selective volatile antimicrobial compounds. Plant Sci. 165: 913-922

Strobel G.A., 2002. Microbial gifts from rain forests. Can J Plant Pathol 24:14-20.

Strobel G.A., Dirkse E., Sears J., Markworth C., 2001. Volatile antimicrobials from *Muscodor albus* a novel endophytic fungus. Microbiology 147: 2943-2950.

Strobel G.A., Hess W.M., 1997. Glucosylation of the peptide leucinostatinA, produced by an endophytic fungus of European yew, may protect the host from leucinostatin toxicity, Chem. Biol. 4: 529-536.

Strobel G.A., Long D.M., 1998. Endophytic microbes embody pharmaceutical potential, ASM News 64 (1998) 263-268.

Strobel G.A., Singh S.K., Riyaz-U-Hassan S., Mitchell A.M., Geary B., Sears J., 2011. An endophytic/pathogenic *Phoma* sp. from creosote bush producing biologically active volatile compounds having fuel potential. FEMS Microbiol Lett 320: 87-94.

Strobel, G.A., 2002. Rainforest endophytes and bioactive products. Criti. Rev. Biotechnol., 22(4):315–333.

Weber D., Sterner O., Anke T., Gorzalczancy S., Martino V., Acevedo C., 2004. Phomol, a new anti-inflammatory metabolite from an endophyte of the medicinal plant *Erythrina crista-galli*, J. Antibiot. 579, 559-563.

Zhigzhitzhapova S.V., Radnaeva L.D., Gao Q., Chen S., Zhang F., 2016. Chemical composition of volatile organic compounds of *Artemisia vulgaris* L. (Asteraceae) from the Qinghai-Tibet Plateau. Industrial Crops and Products, http://dx.doi.org/10.1016/j.indcrop.2015.12.083