academicJournals

Vol. 11(43), pp. 683-689, 17 November, 2017
DOI: 10.5897/JMPR2017.6510
Article Number: 29F933866677
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Antimicrobial evaluation of endophytic fungi extracts isolated from *Casearia sylvestris*

Ernane Gerre Pereira Bastos[1,2*], Aline Aires Aguiar[2], Adriana Idalina Torcato de Oliveira[2], Juliana Fonseca Moreira da Silva[2] and Raphael Sanzio Pimenta[1]

[1]Laboratório de Microbiologia Ambiental e Biotecnologia (LAMBIO), Palmas-TO, Brazil.
[2]Laboratório Microbiologia Geral e Aplicada, Universidade Federal do Tocantins, 77001-923 Palmas-TO, Brazil.

Received 29 September, 2017; Accepted 25 October, 2017

Due to widespread bacterial resistance to commercial antibiotics, the search for capable substances to combating these microorganisms became a priority. In this context, the endophytic fungi gained prominence as potential producers of bioactive substances with pharmacological interest. It is considering that endophytes are still poorly studied, especially in tropical species. The antibacterial and antifungal potential of endophytic fungi associated with the medicinal plant *Casearia sylvestris* were isolated and evaluated. A total of 162 strains were obtained, among these strains, 34 were selected for antimicrobial assays, after molecular sorting with oligonucleotide (GTG)5. A total of 25 isolates showed some antifungal and / or antibacterial activity against the bacteria *Staphylococcus aureus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Escherichia coli*, and yeasts *Candida albicans* and *Candida tropicallis*. The results show the endophytic fungi present in *C. sylvestris* have a high potential to produce bioactive compounds inhibiting pathogenic microorganisms.

Key words: Endophytic fungi, *Casearia sylvestris,* antimicrobial, secondary metabolites.

INTRODUCTION

Endophytic fungi live inside plants colonizing the internal tissues without causing immediate damage to the host. This association suggests that these microorganisms co-evolved with their hosts, presenting an intimate mutualistic relationship, where endophytes receive nutrients and protection while the plant also gains advantages in this interaction, such as greater resistance in environments of intense stress caused by biotic and abiotic factors as insects, herbivores, parasitic nematodes and phytopathogenic microorganisms, water stress and nutrient poor soil (Esposito and Azevedo., 2010; Kharwar et al., 2011; Araújo et al., 2010).

Endophytes associated with the aerial parts of plants have aroused the interest of the scientific community, especially for its potential to produce metabolites of economic interest. Endophytes inhabit a similar ecological niche occupied by phytopathogens, thus being able to control them through competition of nutrients, production of antagonistic substances, parasitizing the pathogen or even inducing the plant to develop resistance (Araújo et al., 2010). Its biotechnological use has been growing in recent decades. They are potentially useful in agriculture and industry, particularly in pharmaceuticals (Souza et al., 2004).

*Corresponding author. E-mail: bastosernane@hotmail.com.

Drug resistance in bacteria has become a global concern and the screening programs for new antibacterial agents are urgent and ongoing. Endophytes provide an abundant reservoir of bioactive metabolites for medicinal exploitation, and an increasing number of novel compounds are being isolated from endophytic fungi. The endophytic fungi natural products present a broad spectrum of activities such as antimicrobial (Aly et al., 2011), antioxidant (Jalgaonwala et al., 2017), immunosuppressive properties, antivirals (Strobel, et al., 2003), anticholinesterase (Aly et al., 2011), antineoplasic (Zhang et al., 2006; Shweta et al., 2010) and cytotoxic activities (Aly et al., 2011). Some studies have shown that endophytic fungi can produce many important bioactive secondary metabolites, such as Taxol (R) from the endophytic fungus *Taxomyces andreanae* and vincristine isolated from the endophytic fungus *Fusarium oxysporum*, which are important anticancer drugs.

The endophytic community may vary by host, geographic distribution, plant age, ecological and seasonal conditions, including altitude and precipitation. Usually only one or two species are dominant as endophytic in a host, while other isolates are uncommon (Arnold et al., 2003). Both the host plant and the region where the endophytes are isolated may influence the secondary metabolite production. In this context, several varieties of plants with pharmacological potentials standout such as *Casearia sylvestris* popularly known as meat leaf, guaçatonga, coffee of bush, wild coffee. It is widely distributed in Brazil and its species excel by the medicinal application. Pharmacological studies have verified in the extracts of this plant antiulcerogenic, anti-inflammatory, antiofidic and cytotoxic activities (Schoenfelder et al., 2008; Santos et al., 2013). Among microorganisms, endophytic fungi have been shown to be prosperous in the production of secondary compounds with pharmacological properties of scientific interest. However, there are still few data in literature about endophytic fungi associated with plant species. Therefore, this study aimed to isolate and evaluate the antibacterial and antifungal properties of endophytic fungi associated with *C. sylvestris* (Figure 1).

MATERIALS AND METHODS

Material collection

The material was collected in Palmas-TO, Brazil, on shores of the lake of the Lajeado hydroelectric power plant. Three leaves from each one of 20 samples of *C. sylvestris* were collected. A sample was recorded in EMBRAPA herbarium (CEN 8047).

Isolation and preservation

The collected leaves were washed previously with neutral detergent and rinsed with sterile water. They were then immersed in 70% (v/v) alcohol (1 min), 2% sodium hypochlorite (3 min) and again in sterile mQ H_2O water (2 min) for final disinfection. Subsequently, five fragments (1.0 cm in diameter) were removed from each leaf and transferred to Petri dishes containing Dextrose-Potato Agar (PDA) medium supplemented with 100 µg /mL chloramphenicol and incubated at 25°C for 60 days. After this period, the grown fungi were isolated and transferred individually to Petri dishes containing PDA and incubated at 25°C for 7 days for purification (Pereira et al., 1993). This procedure ensures the elimination of epiphytic fungi.

Grouping of endophytic fungi

DNA from all isolates was used as a template for amplification by the MP-PCR technique with microsatellite primer (GTG)5 (Lieckfeldt et al. 1993). The products of these amplifications were analyzed on 1.5% agarose gel profiles analyzed in the program PyElph 1.4 (Pavel and Vasile, 2012) with the aim of grouping more similar individuals.

Obtaining crude fungus extracts

The isolated filamentous fungi were grown in PDA at 25°C for 14 days. After this period, fungal mycelium was fragmented with the culture medium, which was subjected to extraction by maceration at room temperature using as extracting liquid a hydroethanolic solution (2:8 v/v). The macerates were filtered, and the crude extracts were evaporated at rotary evaporator to remove the solvent and then lyophilized. Extracts were diluted in 1% dimethyl sulfoxide (DMSO).

Inoculum preparation

Bacterial and fungal suspensions were standardized from a 24 h culture in Mueller Hinton Broth (MHB) for *Staphylococcus aureus* (ATCC 6538), *Listeria monocytogenes* (ATCC 7644), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) bacteria. For yeasts *Candida albicans* (ATCC 10231) and *Candida tropicallis* (ATCC 13803), the Sabouraud broth was used until it reached turbidity equal to the 0.5 tube suspension of the McFarland scale (approximately 1.0×10^8 CFU / mL). The spectrophotometric reading was verified at 620 nm to confirm the concentration of microorganisms. Subsequently, a 1:10 dilution in MHB was performed, obtaining a suspension of 1.0×10^7 CFU / mL, which was used in the tests.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was determined by the microplate dilution technique (96 holes) according to the methodology described according to the M7-A6 standard of the Manual 38 Clinical and Laboratory Standards Institute (CLSI, 2008). The microplate wells were filled with 100 µL of MHB, then 100 µL of fungal extract solution was added and a serial dilution of 1000 to 7.8 µg / mL was performed. In addition, 20 µL of the microorganism suspensions were inoculated into each well of the microplates. As a positive control, chloramphenicol was used for bacterial and fluconazole tests for yeast at the same dilutions as extracts. Control of the culture medium, bacterial growth control and negative control (solvents) were also performed. The microplates were incubated in an incubator at 37°C for 24 h for bacteria and for 48 h for the yeasts; all tests were performed in triplicate.

RESULTS

Grouping of endophytic fungi

The total DNA amplification products of the 162

Figure 1. A. Example of *Casearia sylvestris*. **B.** Highlight of branches with leaves and fruits.

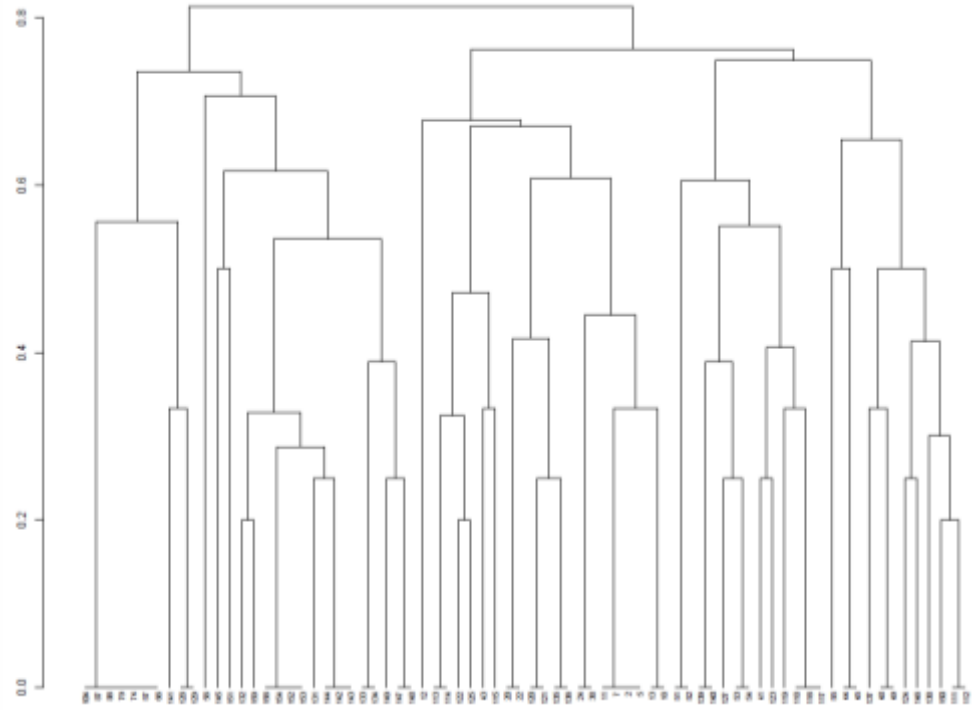

Figure 2. Phylogenetic tree generated by UPGMA method based on DNA amplification of endophytic fungi isolated from *C. sylvestris* and amplified by MSP-PCR with primer (GTG) 5.

endophytic fungus isolates obtained were analyzed and the fingerprint of each isolate was analyzed in the PyElph 1.4 program (Pavel and Vasile, 2012). A primary dendrogram was generated with all individuals. From these grouping analyzes, 34 groups were formed. A second dendrogram was generated excluding individuals with the same amplification profile, so a second dendrogram with better resolution was achieved (Figure 2). From this second grouping, 34 individuals were selected representing each of the groups formed (Table 1).

Antibacterial tests

The antimicrobial activity of ethanolic extracts from each one of 34 fungi is presented in Table 1. Among the extracts tested, 20 (58.8%) showed antibacterial activity against at least one of the four bacteria tested, 14 extracts (41.2%) did not inhibit any of the bacteria. Only one sample (P8B20) inhibited all bacteria tested. *P. aeruginosa* presented the highest resistance against the extracts tested, presenting sensitivity only for 6 (17.6%) samples (P10A2, P11B1, P11C3, P12A5, P13A1,

Table 1. Minimum inhibitory concentration (MIC) of endophytic fungi isolated from *C. sylvestris* against bacteria, *S. aureus*, *L. monocytogenes*, *E. coli*, *P. aeruginosa*.

Endophytic fungi		MIC (µg/ml)			
		Bacteria			
		S. aureus	***L. monocytogenes***	***E. coli***	***P. aeruginosa***
2	P10A2	-	-	1000	125
12	P11B1	-	-	1000	1000
13	P11C3	-	-	-	125
20	P12A4	-	-	-	-
38	P12A5	-	-	125	125
41	P13A1	-	-	125	125
43	P13A2	-	-	-	-
45	P13A4	500	500	250	-
48	P14A1	1000	1000	500	-
51	P14A5	500	1000	500	-
53	P14B1	-	-	-	-
56	P14B3	-	-	-	-
96	P14C1	500	250	125	-
104	P15A1	1000	500	500	-
112	P15A2	-	-	-	-
113	P15B2	-	-	-	-
118	P15B3	-	-	1000	-
121	P16B3	-	-	-	-
122	P16C1	-	-	-	-
124	P16C2	-	-	-	-
127	P17A1	500	250	250	-
129	P17C1	1000	-	500	-
131	P18C2	-	-	-	-
132	P20B2	-	-	-	-
133	P2B6	1000	-	-	-
136	P2C4	1000	-	1000	-
139	P3A3	-	-	-	-
143	P4C4	-	-	-	-
145	P5A3	500	-	-	-
146	P8B2	-	-	250	-
147	P8B20	250	250	125	1000
149	P8B4	-	-	1000	-
150	P8C2	-	-	1000	-
151	P8C3	-	-	-	-

* (-) There was no inhibition at the tested concentrations.

P8B20). *E. coli* presented lower resistance to the tested extracts, presenting sensitivity to 17 (50%) of the samples. The extracts (P10A2, P11B1, P12A5 and P13A1) had antibacterial action only against the Gram-negative bacteria *E. coli* and *P. aeruginosa*. The extracts (P13A4, P14A1, P14A5, P14C1, P15A1 and P17A1) inhibited Gram-positive *S. aureus* and *L. monocytogenes* and at least one Gram-negative, *E. coli*. Isolates (P15B3, P2B6, P8B4, P8C2) showed a limiting MIC in the tests (1000 µg/ml). Isolates P12A5 and P13A1 inhibited *E. coli* and *P. aeruginosa* with the lowest MIC values (125 µg/ml). All extracts tested demonstrated that antibacterial action acted with MIC between 125 and 1000 µg/ml, which demonstrates good or moderate antibacterial activity.

Antifungal tests

The antifungal activity of the ethanolic extracts from each one of 34 fungi tested was evaluated against yeasts *C.*

Table 2. Minimum inhibitory concentration (MIC) of endophytic fungi extracts isolated from *C. sylvestris* against yeasts *C. albicans* and *C. tropicallis*.

Endophytic fungi		MIC (µg/ml) Yeasts	
		C. albicans	***C. tropicallis***
1	P10A2	125	250
2	P11B1	125	1000
3	P11C3	-	-
4	P12A4	250	1000
5	P12A5	250	250
6	P13A1	-	-
7	P13A2	500	500
8	P13A4	500	125
9	P14A1	500	-
10	P14A5	500	-
11	P14B1	-	-
12	P14B3	-	1000
13	P14C1	-	-
14	P15A1	1000	-
15	P15A2	-	-
16	P15B2	-	-
17	P15B3	-	-
18	P16B3	-	-
19	P16C1	-	-
20	P16C2	-	-
21	P17A1	-	-
22	P17C1	-	-
23	P18C2	500	500
24	P20B2	250	125
25	P2B6	1000	500
26	P2C4	1000	500
27	P3A3	-	-
28	P4C4	-	-
29	P5A3	125	-
30	P8B2	500	500
31	P20B8	250	250
32	P8B4	-	-
33	P8C2	-	-
34	P8C3	-	-

* (-) There was no inhibition at the tested concentrations.

albicans and *C. tropicallis.* Of the samples tested, 12 (35.3%) presented antifungal action against the two species of yeasts, 5 extracts (14.7%) presented antifungal action against at least one of the tested yeasts and 17 extracts (50%) showed no antifungal action against yeasts tested. Both yeasts showed similar resistance to the extracts tested (Table 2). The extracts P20B2 and P20B8 inhibited the two yeasts at the lowestinhibitory concentrations tested. The sample P20B8 showed antifungal and antibacterial action for all samples tested. The extracts P12A5, P10A2 and P11B1 showed antimicrobial activity for the yeasts tested and for all Gram-negative bacteria evaluated in this work.

DISCUSSION

The antimicrobial potential of endophytic fungi is associated with their metabolic potential and their ability to produce a great diversity of bioactive molecules, whose main function is to protect the plant from pathogens (Tan and Zou, 2001; Strobel et al., 2003). Many of these bioactive molecules isolated from endophytic belong to various structural classes such as

alkaloids, peptides, steroids, terpenoids, phenols, quinones, and flavonoids (Yu et al., 2010).

Li et al. (2012) reported the presence of acidic, antifungal agent isolated from the endophytic fungi *Pestalotiopsis microspora* and *Monochaetia* sp. present in plant *Torreya taxifolia.* Jadulco et al. (2002) demonstrate the presence of cytosquirins antifungal agent isolated *Curvularia lunata*, present in the plant *Niphates olemda.* Weber et al. (2004) reported the presence of Phomol, which has cytotoxic, antifungal, antibacterial and anti-inflammatory activity and isolated from the endophytic fungi *Phomopsis* spp.

In the present study, 32.3, 50 and 47.1% of the extracts showed antimicrobial activity against *S. aureus*, *E. coli* and *C. albicans*, respectively. These data contrast those of Guimaraes et al. (2008) which examined 39 endophytic fungi extracts and found 5.1, 25.6 and 64% of the extracts inhibited *S. aureus*, *E. coli* and *C. albicans*, respectively, indicating that endophytes of *C. sylvestris* have a similar antibacterial potential as endophytic isolates of *Viguiera arenaria* and *Tithonia*, although it has a lower antifungal action. 11.8% of the tested extracts inhibited only Gram-positive bacteria, a data that corroborate with studies conducted by Ratnaweera et al. (2014) and Philips et al. (1989) which identified an active compound corresponds to the tetramic acid derivative known as equisetin isolated from various species of *Fusarium* spp.; they also demonstrated antimicrobial activity against Gram-positive bacteria such as *B. subtilis*, *S. aureus* and MRSA, but showed no activity against Gram-negative bacteria such as *E. coli*, *P. aeruginosa* and *C. albicans* pathogenic fungi.

The antifungal tests demonstrated that 35.3% of the extracts inhibited *C. albicans* and *C. Tropicallis*. Similarly, Strobel et al. (1999), in studies carried out with extract of fungus *Cryptosporiopsis quercina* isolated from *Tripterigeum wilfordii,* showed antifungal activity against *C. albicans*, which reveals the potential of endophytic fungi to combat this yeast.

In this work, the extracts of 34 endophytic fungi obtained from *C. sylvestris* were tested; 25 of them demonstrated some antibacterial and / or antifungal activity against different Gram positive, Gram negative and yeast microorganisms, by the microdilution technique in plaques; the most recommended method for this determination. The results demonstrate the antibacterial and antifungal properties of these endophytes, revealing the potential of their ethanolic extracts and its application in production of bioactive antimicrobial compounds.

Conclusion

This study reveals that *C. sylvestris* hosts a rich community of endophytic fungi with antimicrobial potential. However, complementary studies are being carried out to identify which secondary components are present in each of the extracts tested.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors thank the Biodiversity and Biotechnology Post Graduate Program of the Amazon (Bionorte), CAPES and Federal University of Tocantins for the financial and logistic support.

REFERENCES

Aly AH, Debbab A, Proksch P (2011). Fifty years of drug discovery from fungi. Fungal Divers. 50(1):3-19.

Araújo WL, Lacava PT, Marcon J, Lima AOS, Sobral JK, Pizzirani-Kleiner AA, Azevedo JL (2010). Guia prático: isolamento e caracterização de microrganismos endofíticos, CALQ-ESALQ: Piracicaba. Available at: http://bdpi.usp.br/single.php?_id=002137784

Arnold AE, Mejia LC, Kyllo D, Rojas EI, Maynard Z, Robbins N, Herre EA (2003). Fungal endophytes limit pathogen damage in a tropical tree. Proceed. Nat. Acad. Sci. 100(26):15649-15654.

Clinical and Laboratory Standards Institute (CLSI) (2008). Approved standard M38-A. Reference method for broth microdilution test for screening of filamentous fungi, Pennsylvania, 48 p. Available at: https://clsi.org/media/1455/m38a2_sample.pdf

Esposito E, Azevedo JL (2010). Fungos uma introdução a biologia, bioquímica e biotecnologia, 2a. ed., EDUCS: Caxias do Sul. Available at: https://www.saraiva.com.br/fungos-uma-introducao-a-biologia-bioquimica-e-biotecnologia-2-ed-3069432.html

Guimaraes DO, Borges WS, Kawano CY, Ribeiro PH, Goldman GH, Nomizo A, Thiemann OH, Oliva G, Lopes NP, Pupo MT (2008). Biological activities from extracts of endophytic fungi isolated from *Viguiera arenaria* and *Tithonia Diversifolia*. FEMS Immunol. Med. Microbiol. 52(1):134-144.

Jadulco R, Brauers G, Edrada RA, Ebel R, Wray V, Sudarsono V, Proksch P (2002). New metabolites from spongederived fungi *Curvularia lunata* and *Cladosporium herbarum*. J. Nat. Prod. 65:730-733.

Jalgaonwala RE, Mohite BV, Mahajan RT (2017). A review: natural products from plant associated endophytic fungi. J. Microbiol. Biotechnol. Res. 1(2):21-32.

Kharwar RN, Mishra A, Gond SK, Stierle A, Stierle D (2011). Anticancer compounds derived from fungal endophytes: Their importance and future challenges. Nat. Prod. Rep. 28(7):1208-1228.

Li Y, Lu C, Huang Y, Li Y, Shen Y Cytochalasin H (2012) A new cytochalasin, isolated from the endophytic fungus *Xylaria* sp. Nat. Prod. Rep. 6(2):121-126.

Lieckfeldt E, Meyer W, Borner T (1993). Rapid identification and differentiation of yeast by DNA and PCR fingerprinting. J. Basic Microbiol. 33:413-426.

Pavel AB, Vasile CI (2012). PyElph-a software tool for gel images analysis and phylogenetics. BMC Bioinformat. 13(9):9.

Pereira JO, Azevedo JL, Petrini O (1993). Endophytic fungi of Stylosanthes: a first report. Mycologia 85:362-364.

Philips NJ, Goodwin JT, Fraiman A, Cole RJ, Lynn DG (1989). Characterization of the Fusarium Toxin Equisetin: The use of Phenylboronates in structure assignment. J. Am. Chem. Soc. 3(21):8223-8231.

Ratnaweera PB, Williams DE, de Silva ED, Wijesundera RLC, Dalisay DS, Andersen RJ (2014). Helvolic acid, an antibacterial nortriterpenoid from a fungal endophyte, *Xylaria* sp. of orchid *Anoectochilus setaceus* endemic to Sri Lanka. Mycology 5(1):23-28.

Santos LS, Rhoden SA, Barros IT, Tonini RCG, Marques RM, Souza VHE, Pamphile JA (2013). A interação harmônica entre fungos e plantas: Aspectos da relação endófito/hospedeiro. Rev. Saúde Biol. 8(1):92-101.

Schoenfelder T, Pich CT, Geremias R, Ávila S, Daminelli EN, Pedrosa RC, Bettiol J (2008). Antihyperlipidemic effect of *Casearia sylvestris* methanolic extract. Fitoterapia 79(6):465-467.

Shweta S, Zuehlke S, Ramesha BT, Priti V, Mohana P, Kumar G, Ravikanth M, Spiteller R, Vasudeva R, Shaanker U (2010). Endophytic fungal strains of *Fusarium solani,* from *Apodytes dimidiata* E. Mey. ex Arn (Icacinaceae) produce camptothecin, 10-hydroxycamptothecin and 9-methoxycamptothecin. Phytochemistry 71(1):117-122.

Souza AQLD, Souza ADLD, Pinheiro MLB, Sarquis MIDM, Pereira, JO (2004). Atividade antimicrobiana de fungos endofíticos isolados de plantas tóxicas da amazônia: *Palicourea longiflora* (aubl.) rich e *Strychnos cogens* bentham. Acta Amazon. 34(2):185-195.

Strobel GA, Miller RV, Martinez-Miller C, Condron MM, Teplow DB, Hess WM (1999). Cryptocandin, a potent antimycotic from the endophytic fungus *Cryptosporiopsis* cf. quercina. Microbiology 145:1919-1926.

Strobel GA, Daisy B (2003). Bioprospecting for microbial endophytes and their natural products. Microbiol. Mol. Biol. Rev. 67(4):491-502.

Tan RX, Zou WX (2001). Endophytes: a rich source of functional metabolites. Nat. Prod. Rep. 18:448-459.

Weber D, Sterner O, Anke T, Gorzalczancy S, Martin V, Acevedo C (2004). Phomol, a new antinflammatory metabolite from an endophyte of the medicinal plant *Erythrina crista-galli*. J. Antibiotics 57:559-563.

Yu H, Zhang L, Li L, Zheng C, Guo L, Li W, Qin L (2010). Recent developments and future prospects of antimicrobial metabolites produced by endophytes. Microbiol. Res. 165(6):437-449.

Zhang HW, Yong CS, Ren XT (2006). Biology and chemistry of endophytes. Nat. Prod. Rep. 23:753-771.