HEALTH FROM FOREST – ANTIOXIDATIVE PROPERTIES OF ENDOPHYTIC FUNGI FROM SCOTS PINE ROOTS

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ABSTRACT

Endophytic fungi are a diverse group of micro-organisms living inside the tissues of host plants. Although the ecological significance of these fungi is not fully understood, they are known to increase plant's fitness and stress tolerance in harsh environmental conditions. They are also a potential source of chemically novel, bioactive compounds for e.g. various medical purposes.

In this study, we monitored antioxidative properties of endophytic fungi living in association with Scots pine roots. On regeneration areas of drained peatlands pine saplings are often subjected to oxidative stress due to extreme temperatures and changing ground water level. It may be expected that the survival of these trees is assisted by endophytic fungi producing antioxidative, protecting compounds.

Altogether 26 mycorrhizal or endophytic fungal strains were isolated from surface sterilized roots of pines and cultivated as pure cultures for four weeks on solid or liquid modified Melin-Norkrans or Hagem medium at room temperature. The fungal tissues were ground with liquid nitrogen and the water soluble compounds were extracted thereafter with water or PBS buffer in a hot water bath. Antioxidative potential of the extracts was screened by determining the ferric reducing ability/antioxidant power (FRAP-test) and the total phenolic content with modified Folin-Ciocalteu's phenol test using microplate applications.

The total phenolic contents of the water-base fungal extracts after protein removal were up to 24±10 mg/g as gallic acid equivalences (GAE) per dry weight of fungus, and the FRAP-values up to 197±11 µmol/g µmol/g as FeSO4 equivalences per dry weight of fungus. The results showed relatively high variation between the fungal isolates in their antioxidant potential. On the other hand, the ferric reducing abilities of the fungal extracts were quite strongly associated with the total phenol test results.

The results show, that the studied endophytic fungal species living in pine roots have antioxidative properties. Additionally, the antioxidative potential of these fungi determined by their ferric reducing ability correlates positively with the production of phenolic compounds.

Keywords: Endophytes; Fungi; Antioxidants

INTRODUCTION

Endophytes are organisms that, during some period of their life, live symptomlessly in the internal tissues of their host plants. In nature, all higher plants are hosts to one or even hundreds of endophytic organisms, which may be fungi, bacteria or actinomycetes. [1, 2]. It has been noticed in laboratory tests, that plants with endophytes are healthier and have more resistance against biotic and abiotic stress than plants without endophytes [3]. The endophytic micro-organisms e.g. produce secondary metabolites to deter herbivores and pathogens, improve resistance to insect pests, enhance thermotolerance of the host plant, or its tolerance to drought, heavy metal presence, low pH or high salinity. They also may produce plant growth promoting substances (phytohormones, cytokines, vitamins, etc.), enhance the host's absorption of nutritional elements, such as N and P, and regulate nutritional qualities, such as the C/N-ratio. A

wide variety of secondary metabolites of endophytic organisms have been reported including alkaloids, steroids, terpenoids, isocoumarin derivatives, quinones, phenylpropanoids and lignans, phenols and phenolic acids, aliphatic compounds, lactones, *etc*. There are also plenties of novel chemical compounds that could be utilized in medical or agricultural purposes such as antibiotics, antioxidant agents or antipathogens [1, 2].

In this study, we monitored the antioxidative potential of a group of endophytic fungi from Scotch pine roots using Folin-Ciocalteu's phenol test and FRAP testing.

MATERIALS AND METHODS

The fungal strains were isolated from the roots of eight year old Scots pines growing on a drained peatland.

Pure cultures of 25 endophytic fungi were isolated from surface sterilized roots onto modified Melin-Norkrans medium (MMN2) [4, 5], or Hagem agar medium [6]. Ectomycorrhizal fungus, *Paxillus involutus* (P.inv.), known to be rich in phenolics, was included among the tested species.

Identification of the fungi isolated from roots was performed by sequencing the amplified ITS-region of the fungal DNA according to White *et al* [7], Gardes and Bruns [8], and Korkama *et al* [9], and comparing the nucleotide sequences to the information in the GenBank.

The fungal cultures were grown for 4 weeks on solid or liquid MMN2 or Hagem medium at room temperature. Agar (15 g/l) was used for solidification of the culture media. There was also a sterilized cellophane membrane on top of the agar medium to prevent the fungus from growing into the agar medium (P 400, Visella Oy, Valkeakoski, Finland). One or three plugs, each 5 mm in diameter and cut from the margin of a 1-month-old fungal colony were then placed on the cellophane membrane on the petri dishes (diameter 9 or 14 cm) and cultivated in the dark at room temperature. Liquid medium, in turn, was used on petri dishes (diameter 9 or 14 cm, liquid volume 25 or 80 ml per dish, respectively) or in bottles (150 ml per bottle). Culturing bottles were stirred 50-100 rpm for aeration.

The fungal mass was collected using a scalpel (solid medium) or by filtering (liquid medium), stored at -20 ºC, and ground with liquid nitrogen in a mortar. About 1 g of each fungal sample was weighed on a petri dish and dried at +45 ºC to determine the percentage of moisture of the samples. The fungal pulps were extracted in sealed polypropylene test tubes (BD Falcon™ or SuperClear™, VWR Collection) with PBS buffer or deionized water in water bath of +95-+100 ºC. One litre of PBS buffer contained 8.0 g NaCl, 0.2 g KCl, 1.42 g $Na_2HPO_4 \cdot 2H_2O$, and 0.23 g KH_2PO_4 in deionized water (reagents from Merck KgaA). Extractions were performed in 2-3 cycles using extractant volumes of 1-15 ml per gram of fresh fungus. Between each cycle the extraction tubes were centrifuged at 6000-7000 g for 10-20 minutes (Eppendorf Centrifuge 5804R, Hamburg, Germany) and the supernatants were collected. In the end the supernatants were mixed and filtered through nylon syringe filters (0.2 µm, Cronus Filter from SMI-LabHut Ltd, Maisemore Gloucester GL2 8EY, UK or 25mm Syringe filter from VWR International, USA). Aliquots of water and PBS buffer without fungal material were extracted simultaneously for control samples.

Antioxidative potential of the extracts was screened by determining the total phenolic content and the ferric reducing ability of the samples with microplate applications of Folin-Ciocalteu's phenol test and FRAP-test, as illustrated below. These tests were performed for original extracts and for extracts, from which proteins were removed by precipitating them (one part) with methanol (four parts) [10]. Protein contents of the samples were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) on microplates based on the Bradford dye-binding procedure [11].

The statistical analyses were performed using SigmaPlot version 11.9 (Systat Software Inc.) and Pearson product moment correlation test (correlation coefficient r).

Phenol testing procedure. The fungal extracts were analyzed for total phenolics using the Folin-Ciocalteu procedure [12] modified for use on 96-well microplates (BD Labware, Franklin Lakes, NJ. USA), as follows.
Gallic acid (Sigma-Aldrich) dilutions of 500, 250, 100 and 25 mg/l in deionized water were prepared freshly each day for standard solutions from a stock solution made by dissolving 500 mg gallic acid in 10 ml of ethanol and diluting with deionized water to 100 ml.

Predilutions of 1/50 from standards and fungal samples were made freshly each day in deionized water. A volume of 160 µl of these dilutions was placed in quadruplicate on a 96-well microplate. One of the quadruplicates was for sample blanking and the three others for color reaction. Additionally, samples of deionized water were placed on each plate in quadruplicate for blanking the color reagent. The final values were thus corrected for background absorbances caused by either the sample or the reagents. As testing samples treated with methanol for protein removal, the standard dilutions and the water blank for the phenol test were treated with methanol similarly.

For the sample blanks, 40 µl of 20% Na_2CO_3 (Merck KGaA) solution was added. Then, 10 µl of the Folin-Ciocalteu's phenol reagent (Merck KGaA) was added for the color reaction samples and the microplate was shaken for one minute. After five minutes, 30 µl of 20% Na_2CO_3 solution was added to the color reaction samples, and the plate was shaken again for one minute. The absorbance at 750 nm was monitored by a microplate reader (Multiskan RC, Labsystems, Finland) after one hour of incubation at room temperature from addition of the Na_2CO_3 solution. The plate was shaken just before the measurement for one minute. A lag time of 15 seconds was used between the measurements of each plate columns. The phenol test results are expressed as gallic acid equivalences, mg/g dry weight of fungus (GAE).

FRAP testing procedure. FRAP assay of fungal samples was performed using a modified version of the method described by Firuzi *et al* [13], as follows.

Dilutions of 1000, 500, 200, and 50 µM of ferrous sulphate for standard solutions were prepared in deionized water freshly each day from a stock solution of 10 mM. The stock solution was also prepared freshly for each day by dissolving 0.1390 g of $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich) in 50 ml of deionized water.

For FRAP color reagent, acetate buffer (300 mM, pH 3.6, reagents from Merck KgaA), ferric chloride (20 mM, $FeCl_3 \cdot 6H_2O$ from Sigma-Aldrich), and TPTZ-solution of 10 mM [2,4,6-Tris(2-pyridyl)-s-triazine from Sigma-Aldrich] were mixed in proportion of 4:1:1. The mixture was made freshly for each day.

For acetate buffer of 300 mM (pH 3.6), 1.68 g of anhydrous sodium acetate was dissolved in 800 ml of deionized water, and 16 ml of acetic acid was added. The total volume was added to 1000 ml with deionized water. The pH was corrected with acetic acid or with 0.1 N NaOH, as needed.

Ferric chloride of 20 mM was made by dissolving 270 mg FeCl3·6H2O to 50 ml of deionized water, and for TPTZ-solution of 10 mM, 160 mg of TPTZ was dissolved in 50 ml of 40 mM HCl.

A volume of 25 µl of standard dilutions and fungal samples was added on a 96-well microplate (BD Labware, Franklin Lakes, NJ. USA) in quadruplicates. One of these aliquots was for sample blanking and the three others for color reaction. Additionally, samples of deionized water were placed on each plate in quadruplicate for blanking the color reagent. The final values were thus corrected for background absorbances caused by either the sample or the reagents. As

testing samples treated with methanol for protein removal, the standard dilutions and the water blank for the FRAP test were treated with methanol similarly.

Thereafter, 50 µl of acetate buffer (300 mM) was added to each well. For the sample blanks, an additional 100 µl of the same acetate buffer was added. For standards diluted in water and for water extracted fungal samples, 25 µl PBS buffer was added. For the fungal samples extracted with PBS buffer, in turn, 25 µl of deionized water was added to equalize the matrix effects in the reaction mixtures.

Finally, 100 µl of the FRAP reagent was added to the color reaction wells of the plate, and the plate was shaken for 30 seconds. The absorbances were read after 4 minutes at 590 nm using a lag time of 7 seconds between the plate columns.

RESULTS AND DISCUSSION

DNA sequences of 16 fungus isolates of the 26 (Table 1) showed matching with Genbank database on species or family level. Antioxidant potential showed relatively high variation between the fungal isolates. Also the type of growth medium used for culturing, as well as the extractant affected the results markedly (Fig. 1A-D). The highest total phenolic contents [24 ± 10 mg/g gallic acid equivalences (GAE) per dry weight] and FRAP values [197 ± 11 µmol/g as $FeSO_4$ equivalences ($FeSO_4$ eq.) per dry weight] were measured from PBS buffer extract (without proteins) of *Articulospora* sp. (code N) (Fig. 1B).

The protein contents correlated positively with the phenolic test results of the original extracts, but not with the ones after protein removal (Fig. 2A). On the other hand, the FRAP values of extracts before and after protein removal correlated positively with protein contents (Fig. 2B). Methanol used for protein precipitation seemed to affect the phenol and the FRAP test results by increasing the values as seen in the Fig. 2A and Fig. 2B, which is in line with earlier reports e.g. by Firuzi *et al* [13]. Also the Folin-Ciocalteu's phenol test is known to be interfered by several factors [12].

The ferric reducing abilities of the fungal extracts were strongly associated with the phenol test results. This was the case both in the samples including proteins (data not shown, $r = 0.688$, $p < 0.001$, $n = 22$) and in the samples after protein removal (Fig. 3, $r = 0.652$, $p < 0.01$, $n = 22$). Similar correlation between phenol contents and FRAP values has been shown to exist also e.g. in medicinal plant extracts [14].

Table 1: The species or the family names of the fungi under investigation according to the information of the GenBank.

Fungus code	Species or family name	Fungus code	Species or family name
A	Acephala applanata	S15	Ascomycete clones
B	unknown	S16	unknown
C	unknown	S17	unknown
D	Phialophora lignicola	S18	Meliniomyces variabilis
E	unknown	S19	Pezizomycotina
F	unknown	S20	Pezizomycotina
G	Dermea sp.	S21	unknown
H	unknown	S22	unknown
L	Ascomycete clones	S23	Phialocephala sp.
M	Umbelopsis sp.	S24	Ascomycete clones
N	Articulospora sp.	S25	Phialocephala fortinii
R	Phialocephala sp.	S26	unknown
		S27	Penicillium sp.
		P. Inv	Paxillus involutus (Ectomycorrhizal fungus)

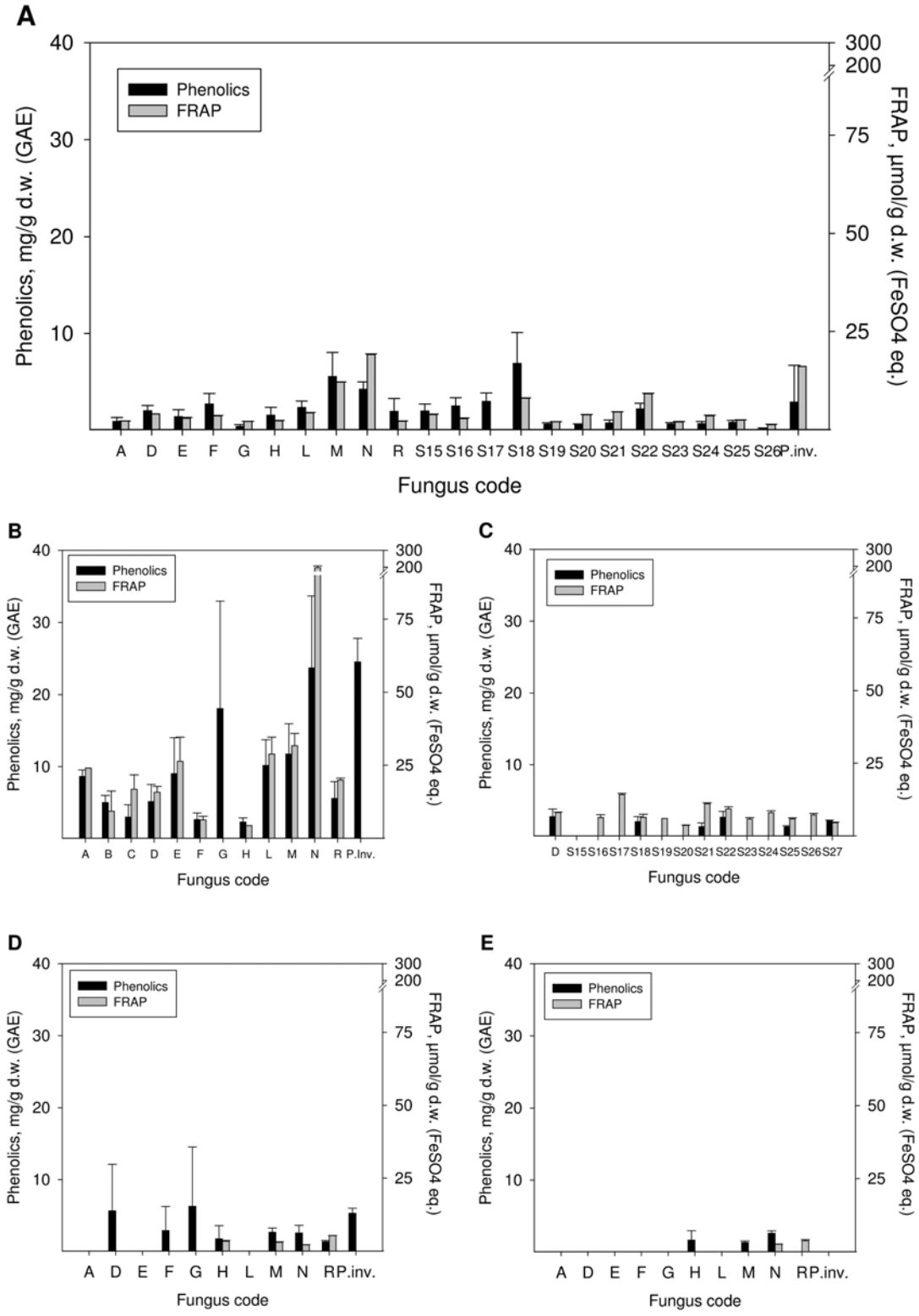

Figure 1: The phenolic contents (mg GAE/g dry weight) and FRAP values (µmol $FeSO_4$ eq./g dry weight) of fungal extracts without protein (methanol precipitation). (A) Fungi from solid Hagem plates, water extraction. (B) Fungi from solid MMN2 plates, PBS buffer extraction. (C) Fungi from solid MMN2 plates, water extraction. (D) Fungi from liquid Hagem plates, water extraction. (E) Fungi from liquid MMN2 plates, water extraction. The results are expressed as mean of triplicate measurements ± SD.

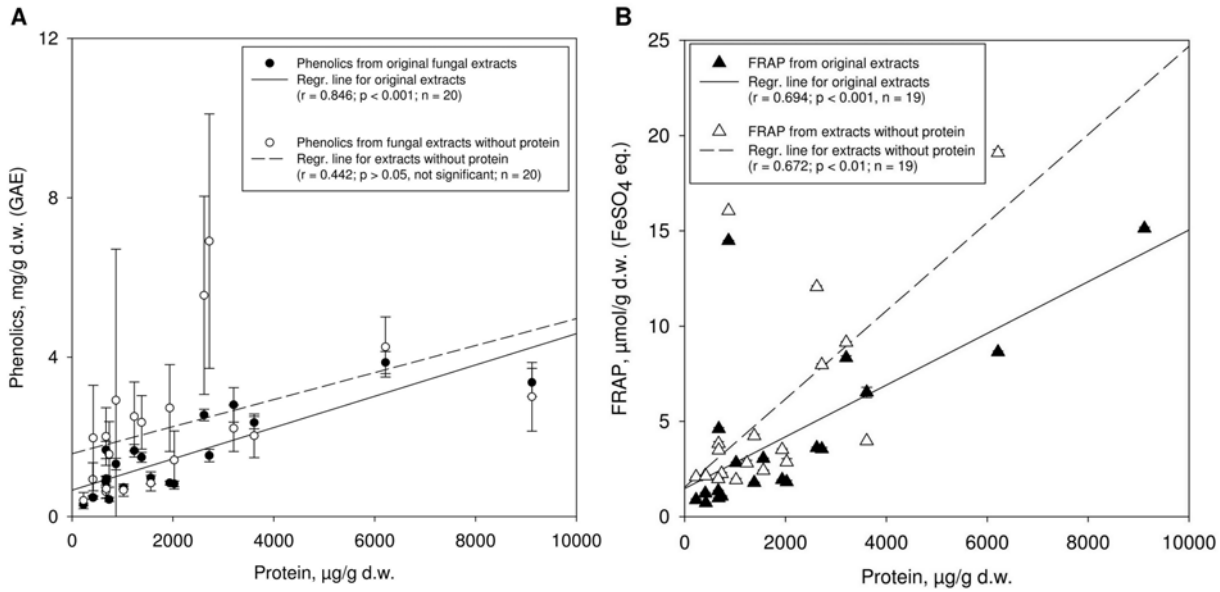

Figure 2: The mean protein contents of the original fungal water extracts (fungi from solid Hagem plates) versus (A) the phenolic contents of the original extracts and of the extracts precipitated with methanol to remove protein with the corresponding regression lines, and (B) the FRAP values of the original extracts and of the extracts precipitated with methanol to remove protein and the corresponding regression lines. The Pearson coefficients for correlation (r), significances (p) and number of samples (n) are found in the figure legends. The results of y-axels are expressed as mean of triplicate measurements ± SD.

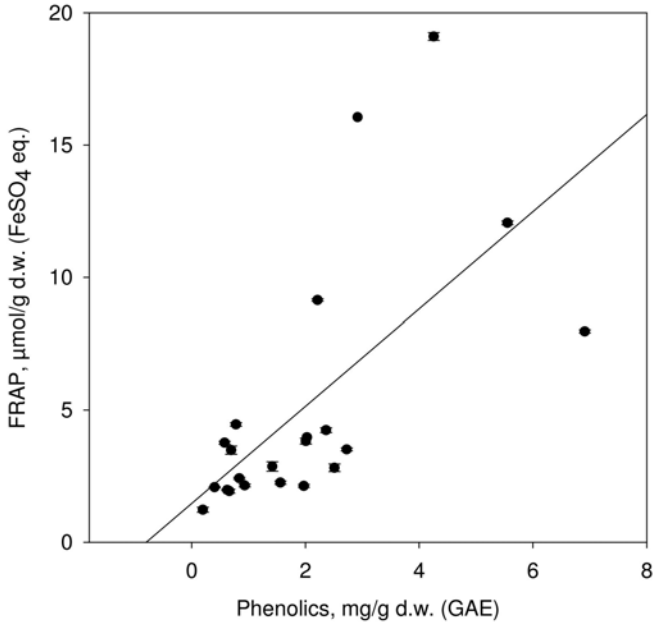

Figure 3: The mean phenolic contents versus the FRAP values with the corresponding regression line of the fungal water extracts (fungi from solid Hagem plates) precipitated with methanol to remove protein (r= 0.652, p < 0.01, n = 22). The FRAP results are expressed as mean of triplicate measurements ± SD.

CONCLUSIONS

The results show, that the fungal endophytes living in pine roots have antioxidative properties. Additionally, the antioxidative potential of these fungi related to their ferric reducing ability, appears to correlate with the production of phenolic compounds. This suggests that phenolics

may function as antioxidants in the studied endophytic fungi. The correlations between ferric reducing abilities of the fungi with the amount of proteins show that protein structures may be linked to the mechanisms of antioxidative properties, but not necessarily as functional components.

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