Arch Biol Sci. 2016;68(2):279-289 DOI:10.2298/ABS150602016J

ASPERGILLUS PIPERIS A/5 FROM PLUM-DISTILLING WASTE COMPOST PRODUCES A COMPLEX OF ANTIFUNGAL METABOLITES ACTIVE AGAINST THE PHYTOPATHOGEN *PYTHIUM APHANIDERMATUM*

Jelena Jovičić-Petrović[1,*], Sanja Jeremić[2], Ivan Vučković[3], Sandra Vojnović[2], Aleksandra Bulajić[1], Vera Raičević[1] and Jasmina Nikodinović-Runić[2]

[1] *Faculty of Agriculture, University of Belgrade*, Nemanjina 6, 11080 Zemun, Belgrade, Serbia
[2] *Institute of Molecular Genetics and Genetic Engineering, University of Belgrade*, Vojvode Stepe 444a, 11000 Belgrade, Serbia
[3] *Faculty of Chemistry, University of Belgrade*, Studentski trg 12-16, 11000 Belgrade, Serbia

***Corresponding author:** jelenap@agrif.bg.ac.rs

Received: June 2, 2015; **Revised**: August 4, 2015; **Accepted**: September 1, 2015; **Published online**: March 8, 2016

Abstract: Adding compost to soil can result in plant disease suppression through the mechanisms of antagonistic action of compost microflora against plant pathogens. The aim of the study was to select effective antagonists of *Pythium aphanidermatum* from compost, to assess the effect of its extracellular metabolites on the plant pathogen, and to characterize antifungal metabolites. The fungal isolate selected by a confrontation test was identified as *Aspergillus piperis* A/5 on the basis of morphological features and the internal transcribed spacer (ITS) region, β-tubulin and calmodulin partial sequences. Liquid chromatography-mass spectroscopy (LC-MS) analysis showed that gluconic and citric acid were the most abundant in the organic culture extract. However, the main antifungal activity was contained in the aqueous phase remaining after the organic solvent extraction. The presence of considerable amounts of proteins in both the crude culture extract as well as the aqueous phase remaining after solvent extraction was confirmed by SDS-PAGE. Isolated *Aspergillus piperis* A/5 exhibits strong antifungal activity against the phytopathogen *Pythium aphanidermatum*. It secretes a complex mixture of metabolites consisting of small molecules, including gluconic acid, citric acid and itaconic acid derivatives, but the most potent antifungal activity was associated with proteins resistant to heat and organic solvents. Our findings about the activity and characterization of antagonistic strain metabolites contribute to the understanding of the mechanism of interaction of antifungal metabolites as well as fungal-fungal interaction. The obtained results provide a basis for further application development in agriculture and food processing.

Key words: antifungal activity; antifungal proteins; *Aspergillus piperis*; organic acids; *Pythium aphanidermatum*; compost.

INTRODUCTION

Growing concerns regarding the sustainability requirements, environmental quality, human health and plant pathogen resistance to conventional pesticides have resulted in the need to find new approaches to plant protection [1,2]. Among others, an integrated approach in plant protection involves the use of residue management and compost as a soil organic amendment [3-5].

Many filamentous fungi are destructive pathogens of plants and are thus responsible for enormous crop losses worldwide. *Pythium aphanidermatum* is a widespread soil-borne plant pathogen and a member of the class *Oomycetes* [6]. It causes diseases in multiple plant species, leading to significant losses in agriculture [7]. New hosts and diseases of this pathogen are continuously reported and it has recently been also associated with human invasive wound infection [8]. *P. aphanidermatum* is susceptible to different biotic and abiotic factors during its saprophytic phase in soil [9]. Biotic factors include interactions with other microorganisms in soil, and *P. aphanidermatum* sensitivity to competition and antagonism may be the key potential for its biological control [4,10].

Soil amendment with compost is an agricultural practice commonly used to improve soil quality and manage organic wastes [11]. Recycling composted organic residues in agriculture can reduce the need for mineral fertilizers and improve the physicochemical and biological properties of cultivated soils [2,12]. The microbial community contributes to the soil and compost suppressiveness, but disease suppression may be based on the activity of a small proportion of the total biomass [13,14]. There are numerous examples of soil-borne pathogens controlled effectively by the application of compost as an organic amendment including suppression of *Pythium* species [15-17]. On the other hand, filamentous fungi are known as important inhabitants of compost and a rich source of bioactive compounds, with many of them identified as plant pathogen antagonists [18]. Compost produced in the open air (field compost), has been known for its high biocontrol potential for a long time [19].

In this study, we explored field compost from agro-industrial waste as a source of efficient phytopathogen fungal antagonists. Our aim was to select effective antagonists of *P. aphanidermatum* and to study and characterize their bioactive metabolites against this harmful plant pathogen.

MATERIALS AND METHODS

Strains and media

P. aphanidermatum and *Botrytis cinerea* cultures were from the collection of the Institute for Pesticides and Environmental Protection, Belgrade, Serbia, and *Fusarium oxysporum* was from the Culture Collection of the Institute for Phytomedicine, Faculty of Agriculture, Belgrade, Serbia. *P. aphanidermatum* culture was maintained on potato dextrose agar (PDA; Sigma Aldrich®, Saint Louis, USA) at 25°C and transferred to fresh media every week. *B. cinerea* and *F. oxysporum* were maintained on PDA at 4°C and subcultured every month. Potential fungal antagonists of *P. aphanidermatum* were isolated from mature compost made of stillage derived from plum-brandy production. Fungi were isolated by serial dilution technique on selective media, Rose Bengal Agar with streptomycin (30 μg mL^{-1}) [20]. The obtained monosporic cultures were maintained at 4°C on PDA. Selected isolates were cultivated in potato dextrose broth (PDB; Sigma Aldrich®, Saint Louise, USA) for metabolite production and characterization.

Selection of antagonistic fungi by confrontation assay

Selection of plant pathogen antagonists was performed by confrontation test as previously described [21]. Briefly, disks (5-mm diameter) obtained from the edge of actively growing colonies of plant pathogens and a specific isolate from compost were placed 3 cm apart on a PDA plate. The interaction between the two colonies was observed after incubation at 25°C in the dark, and the appearance of the colony of pathogens was also monitored and recorded. Incubation time was 3, 5 and 7 days for *P. aphanidermatum*, *B. cinerea* and *F. oxysporum*, respectively. The colony diameter of the plant pathogen was measured and compared to that of the control, which was the diameter of plant pathogen grown in pure culture on PDA plates. The test was conducted in three replications in two independent experiments. Inhibition percentage was determined as: (colony diameter of control – colony diameter after treatment)/colony diameter of control × 100.

Taxonomic identification of the fungal isolate

Morphological identification of the antagonistic fungal isolate was performed on the basis of colony appearance and microscopic examination (Leica DMLS, Leica Microsystems GmbH, Wetzlar, Germany) on 7-day-old culture grown on PDA at 25°C in darkness.

Molecular identification was carried out by amplification and sequencing of three genes (18S ITS region, β-tubulin and calmodulin). Fungal DNA was extracted by DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and amplifications were conducted in 25 μL reaction volumes containing 12.5 μL of Master Mix (Fermentas, Vilnius, Lithuania), 10 pmol of each primer, 100 ng of DNA and 6.5 μL of RNase-free wa-

ter, in Thermocycler T-1 (Biometra, GmbH, Germany). The ITS region was amplified using an ITS1/ITS4 primer pair [22], the β-tubulin region was amplified using a Bt2a/Bt2b primer pair [23], and the calmodulin region was amplified using CL1/CL2A primer pair according to the previously reported protocol [24].

Amplified products were visualized by 1% [w/v] agarose gel electrophoresis. Polymerase chain reaction (PCR) products were sequenced on an ABI 3730XL Sequencer (Macrogen, Inc., Seoul, Korea) in both directions using the same respective primer pairs, and the BLASTN program (NCBI, http://www.ncbi.nlm.nih.gov) [25] was used for sequence similarity searches. Alignment of the obtained ITS sequence (586 bp), partial β-tubulin (524 bp) and calmodulin (653 bp) sequences and homologous sequences taken from GenBank was performed by Clustal W 2.0 algorithm [26]. A phylogenetic tree was constructed by the maximum-likelihood algorithm using Tamura-Nei distance correction and Bootstrap resampling method, all included in the MEGA6 package [27]. The phylogenetic tree was rooted using ITS, partial β-tubulin and calmodulin sequences of *Aspergillus robustus* NRRL 6362^{T} as an outgroup. Sequences obtained within this research were deposited in the GenBank database with accession numbers: KF991390 (ITS sequence), KJ469441 (β-tubulin), and KP019623 (calmodulin A).

Aspergillus piperis A/5 maintenance, growth, crude culture filtrate and extract preparation

The A/5 isolate was precultured on PDA in darkness at 25°C for 7 days. For crude culture filtrate (CCF) preparation, A/5 was grown in PDB. Flasks containing 200 mL of sterile PDB were inoculated with five 5-mm-diameter mycelial disks obtained from the edge of the colony and incubated in darkness at 25°C for 7 days on a rotary shaker (Environmental Shaker Incubator ES-20, BIOSAN, Riga, Latvia) at 160 rpm. Liquid culture was filtered through sterile cheese cloth and then centrifuged at 25°C at 13000 × g (Centrifuge U-320, BOECO, Hamburg, Germany). The supernatant was carefully collected and sterilized by filtering through a 0.45-μm Millipore membrane (Merck KGaA, Darmstadt, Germany) to obtain CCF. In some cases, the CCF was autoclaved at 121°C for 15 min.

The CCF was subjected to extraction with an equal volume of ethyl acetate by vigorous shaking at 25°C, 150 rpm for 12 h. Ethyl acetate extract was separated from the remaining aqueous phase (AP) using a separating funnel, evaporated under reduced pressure and weighed. This crude culture ethyl acetate extract (CCE) was dissolved in dimethyl sulfoxide (DMSO) and used in antimicrobial screening. Any remaining ethyl acetate was removed from the AP under reduced pressure and was further used in antimicrobial screening.

A qualitative enzyme assay of the CCF and AP was conducted by API ZYM (Biomerieux, Marcy l'Etoile, France) according to the manufacturer's instructions.

Treatment of CCF and AP with proteinase K

The pH values of the CCF and AP were measured electrochemically (CyberScan Ion 510, Eutech Instruments, Singapore), and then adjusted to 4.5 by addition of NaOH to 0.1 mol L^{-1}). Subsequently, CCF and AP were treated with 0.1 mg mL^{-1} of proteinase K (Sigma, Saint Lois, USA) for 1 h at 37°C. The enzyme was inactivated by boiling for 15 min. Inhibition of *P. aphanidermatum* was tested in the presence of CCF and AP in following treatments: original CCF and AP, CCF and AP with pH adjusted to 4.5 and CCF and AP treated with proteinase K.

Antifungal activity screening

Inhibition of *P. aphanidermatum* by the CCF and fractions obtained after ethyl acetate extraction (CCE and AP) was assessed by dissolving appropriate amounts in PDA medium. PDA medium melted at 45°C was amended with sterile CCF, AP and CCE in a comparable range of concentrations of 2-10% (v/v). The 5-mm-diameter disks cut from the edge of the 2-day-old colony of *P. aphanidermatum* were plated centrally on media containing filtrate or extract. Plant pathogen growth on PDA without CCF was used as a control. The experiment was conducted in three independent

replicates. After two days of incubation at 25°C in the dark, the colony diameter was measured.

Protein separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins in CCF (10 mL) were precipitated by trichloroacetic acid (TCA) according to the the standard protocol [29]. This procedure was also used for the precipitation of proteins from the AP after the ethyl acetate extraction of the supernatant of *A. piperis* A/5 culture, but the starting volume was doubled for adequate comparison (20 mL). In both cases, pelleted proteins were resuspended in 0.5 mL of 20 mM phosphate buffer pH 6.5 and additionally concentrated (5x) using Microcon Centrifugal Filters-cut off 10 kDa (Millipore, Merck KGaA, Darmstadt, Germany). Protein concentration was determined using the standard Bradford assay [30]. Prepared samples (25 µL) were analyzed by SDS-PAGE [31]. The polyacrylamide concentration in the separating gel was 12% (w/v). The Amersham Low Molecular Weight Calibration Kit for SDS Electrophoresis (GE Healthcare Buckinghamshire, UK) was used as a protein molecular size standard. Proteins were stained with Coomassie Brilliant Blue R 250 (Sigma, Saint Lois, USA).

Chemical analysis of *A. piperis* A/5 extracellular metabolites

Citric acid, gluconic acid, succinic acid and other chemicals were purchased from Sigma-Aldrich (Saint Lois, USA). LC-MS analysis was performed on the CCF, AP and CCE using the HPLC instrument Agilent 1200 Series (Agilent Technologies, Santa Clara, USA) with a Zorbax Extend C18 column (RR HT 50 × 4.6 mm, 1.8 µm) and a diode-array detector (DAD), coupled with a 6210 Time-of-Flight LC/MS system (Agilent Technologies, Santa Clara, USA). The column temperature was 40°C with a constant flow rate of 0.5 mL min^{-1}. The mobile phase was a gradient prepared from 0.2% formic acid in water (A) and acetonitrile (B), according to the following program: 0-0.24 min, 5% B; 0.24-10 min 5-95% B; 10-15 min 95% B; 15-15.5 min 95-5% B; 15.5-18.5 min 5% B. High resolution electrospray ionisation mass spectrometry (ESI-MS) spectra were recorded in the range of 100-2500 *m/z* in both positive and negative ion mode, with 4000 V ion source potential and 70 V fragmentor potential.

Statistical analysis

Collected data were subjected to analysis of variance (ANOVA) by the software Statistica (StatSoft, Tulsa, OK, USA). Mean values of data were compared by Fisher's LSD test at a significance level of P=0.05.

RESULTS

Selection of antagonistic fungal isolates from the compost heap sample

Pure cultures of 14 morphologically different isolates representing dominant compost fungi were obtained and tested to select for efficient antagonists of *P. aphanidermatum*. Results of the confrontation test showed that the isolate A/5 produced the highest inhibition percentage of *P. aphanidermatum* (81%), and was chosen for further study (Fig. 1a). The same isolate inhibited *B. cinerea* growth and completely overgrew its colony, while in a paired culture plate assay using *F. oxysporum* it induced colony diameter reduction on average by 33%. Based on the most efficient antagonistic activity against *P. aphanidermatum*, as well as exhibited activity against other phytopathogens, the isolate A/5 was selected for further study.

Isolate A/5 identification and characterization

The morphological features of the isolate A/5 were consistent with common features of *Aspergillus* sp., a member of section *Nigri*. The isolate was characterized by creamy white substrate mycelia with sparsely produced black conidial areas, transparent conidiophores, oval vesicles bearing transparent one-row phialides with conidia 3-4 µm in diameter (Fig. 1a and 1b). Upon 7 days' incubation in liquid medium, the culture broth from pH 6.8 reached pH 2. Its maximal growth temperature was 45°C, indicating that it could

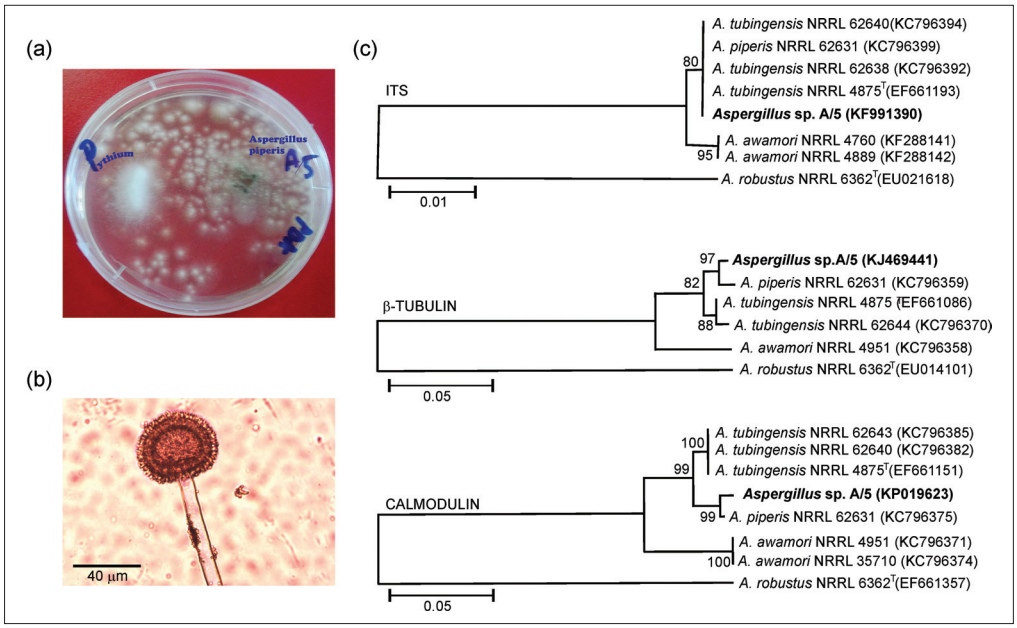

Fig. 1. Fungal isolate *A. piperis* A/5; **a** – on PDA medium in confrontation assay against *P. aphanidermatum* (left side of the plate); **b** – micrograph of 100 × magnification; **c** – maximum-likelihood phylogenetic tree based on ITS, partial β-tubulin and calmodulin sequences, showing the phylogenetic relationship of isolate *Aspergillus* sp. A/5 (presented in bold) and closely related strains, using *A. robustus* NRRL 6362^T as an outgroup. Bootstrap values at branch points are expressed as a percentage of 1000 replications. GenBank accession numbers are in brackets. The scale bar represents 0.01 and 0.05 substitutions per nucleotide position, for ITS and β-tubulin/calmodulin partial sequences, respectively.

survive the composting conditions in the outer layers of a compost heap. On the basis of these features, the A/5 isolate was assigned as a member of the *Aspergillus niger* 'aggregate', which consists of morphologically indistinguishable taxa: *A. niger*, *A. tubingensis*, *A. acidus*, *A. brasiliensis*, *A. costaricaensis*, *A. lacticoffeatus*, *A. piperis* and *A. vadensis* [32]. Section *Nigri* consists of 26 closely related species that are hardly distinguishable by morphological properties [33].

Based on the ITS sequences, A/5 strain was also placed in the genus *Aspergillus,* and grouped with *A. tubingensis* and *A. piperis* strains. In the case of β-tubulin and calmodulin partial sequences, strain A/5 was unequivocally clustered with *A. piperis* NRRL 62631^T, based on 99% sequence identity. These results are in accordance with the search results of the BLAST NCBI nucleotide collection (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Strain A/5 was identified as *A. piperis* based on molecular identification and morphological features that are consistent with the description of *A. piperis* given previously [34].

Inhibition of *P. aphanidermatum* by *A. piperis* A/5 extracellular metabolites

Microbial extracts represent a good source of biotechnologically important new molecules. *Aspergillus* and *Penicillium* species are known as producers of a large number of useful metabolites, especially those with antimicrobial activity [35]. Indeed, the CCF of *A. piperis* A/5 exhibited significant activity against *P. aphanidermatum*. CCF in a concentration of 10% (v/v) completely inhibited the growth of the plant pathogen, while at lower concentrations of 2% (v/v) it stimulated *P. aphanidermatum* growth (Table 1). Surprisingly, the AP exhibited much higher activity in comparison to the CCF with 2% (v/v) inhibiting *P. aphanidermatum* by 92% (Table 1). Both the CCF and AP completely

Table 1. Inhibition of *P. aphanidermatum* growth using *A. piperis* A/5 crude culture filtrate (CCF) and aqueous phase (AP) after ethyl acetate extraction of small organic metabolites in PDA dilution assay.

Treatment	Inhibition percentage (%)[a]
CCF	
10%	100
5%	75±3
4%	74±2
2%	-4.5±1[b]
AP	
10%	100
5%	100
4%	100
2%	92±2

[a]mean values of three independent experiments; [b]stimulation of fungal growth

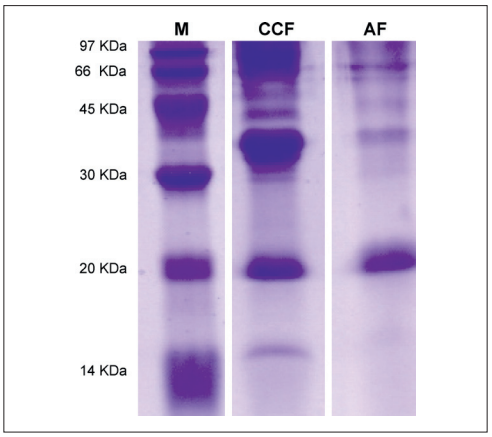

Fig. 2. SDS-PAGE of proteins precipitated from *A. piperis* A/5 crude culture filtrate before (CCF) and after solvent extraction (AP). Lane M, protein molecular mass marker.

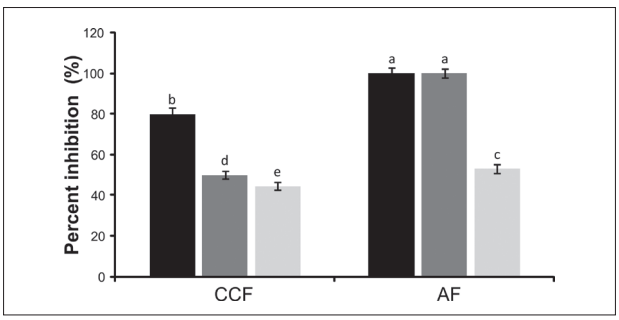

Fig. 3. Growth inhibition of *P. aphanidermatum* in presence of crude culture filtrate (CCF) and aqueous phase after ethylacetate extraction of small organic metabolites (AP); ■, CCF and AP with pH adjusted to 4.5 (■); and CCF- and AP-treated with proteinase K (■). Mean values with the same letter are not significantly different according to Fisher's LSD test (p= 0.05).

retained their activities after autoclaving. The difference in CCF and AP activity could be attributed to the presence of small organic molecules, predominantly organic acids in the CCF that were removed during solvent extraction. From 100 mL of *A. piperis* A/5 CCF, on average 12.0 mg of ethyl acetate extract was obtained. This fraction contained gluconic and citric acid amounting to 90% (w/w) and some other organic acids, including succinic, muconic and itaconic acids (results not shown). Species of the genus *Aspergillus* section *Nigri* are known as organic acid producers in biotechnology. They are mainly used in the production of gluconic and citric acid [36]. Nevertheless, CCE, which predominantly consisted of organic acids, had no antifungal activity. Thus, from these results it could be concluded that the main bioactive metabolites in *A. piperis* were proteins or peptides, and that the presence of organic acids affected their activity.

Proteins were detected in CCF and AP (Fig. 2). The 7-day-old CCF of *A. piperis* A/5 usually contained 6.8-10.2 mg mL^{-1} of proteins, while after ethyl acetate precipitation, the amount of protein remaining was between 1.8-2.5 mg mL^{-1} (Fig. 2). On the other hand, the pH values of both CCF and AP were 2.0. To further assess the activity of CCF and AP, we raised their pH to 4.5 and treated both with proteinase K. The AP completely retained its antifungal activity after pH elevation to 4.5, while the effect of the CCF was reduced by 30%. Treatment of the AP treatment with protease K decreased its inhibitory effect on *P. aphanidermatum* growth almost two-fold; despite this, addition of this AP still led to significant inhibition of the phytopathogen. Protease K treatment reduced the inhibitory effect of the CCF, but only by 5.5% in comparison to the effect of CCF with adjusted pH (Fig. 3).

The qualitative enzyme activity test of the CCF showed strong activity of alkaline and acid phosphatases, phosphohydrolase and α-galactosidase, moderate activity of N-acetyl-β-glucosaminidase

Table 2. Qualitative enzyme screening of *A. piperis* A/5 crude culture filtrate (CCF) and aqueous phase (AP) after ethyl acetate extraction.

Enzyme	CCF	AP
Alkaline phosphatase	+++[a]	++
Esterase[C4]	-[b]	-
Esterase lipase [C8]	-	-
Lipase [C14]	-	-
Leucine arylamidase	-	-
Valine arylamidase	-	-
Cystine arylamidase	-	-
Trypsin	-	-
α-chymotrypsin	-	-
Acid phosphatase	+++	-
Naphthol-AS-phosphohydrolase	+++	-
α-galactosidase	+++	-
β-galactosidase	+	-
β-glucuronidase	-	-
α-glucosidase	-	-
β-glucosidase	-	-
N-acetyl-β glucosaminidase	++	-
α-mannosidase	-	-
α-fucosidase	-	-

[a]+, weak positive activity; ++, positive activity; +++, strong positive activity; [b]-, absence of activity

and weak activity of β-galactosidase (Table 2). The only activity detected in the AP was that of alkaline phosphatase (Table 2). N-acetyl-β-glucosaminidase is a lytic enzyme produced by numerous fungi and has been associated with plant pathogen inhibition [37]. Considering that no N-acetyl-β-glucosaminidase activity was detected in the AP (Table 2), the enhanced AP activity was most likely due to some other proteinaceous compound with a lower molecular weight.

The predominant protein bands in the CCF were 20 kDa and 38 kDa, while the most abundant protein in the AP was about 20 kDa (Fig. 2). This protein was solvent-tolerant and possibly involved in the antifungal activity of the AP. Representatives of genus *Aspergillus*, including *A. niger*, were previously reported to produce bioactive proteins [38-41]. However, these peptides are about 10-15 kDa, and we did not detect this particular fraction amongst precipitated proteins from *A. piperis* A/5 CCF or AP (Fig. 2).

Chemical characterization of extracellular metabolites

The crude culture filtrate and the aqueous phase remaining after the extraction of small organic molecules still retained a pH of 2 and were subjected to chromatographic separation and analysis (Fig. 4). The metabolic profiles of CCF and AP were comparable, with several organic acids identified in both (Fig. 2). The identification was based on high-resolution ESI-MS spectra recorded in positive and negative ion mode. The major components in CCF were gluconic acid and citric acid, and glucose acid, tartaric acid, succinic acid and muconic acid were also present (Fig. 2a). In addition, two compounds corresponding to the molecular formulae $C_{11}H_{18}O_5$ (RT 4.62 min) and $C_{11}H_{16}O_6$ (RT 4.70 min) were detected. They could be attributed to itaconic acid derivatives, the same or similar to those previously isolated from a seawater isolate of *A. aculeatus*, namely 9-hydroxylitaconic acid and 9-hydroxylitaconic acid-4-methyl ester [42]. Apart from citric acid, the AP contained considerably lower amounts of all acids in comparison to the CCF (Fig. 4b). In both fractions, the presence of polypeptide_ component was detected, based on the characteristic ESI-MS spectrum recorded in positive ion mode [results not shown].

DISCUSSION

We have shown that field compost from agricultural waste is a good source of new organisms that can possibly be used as biocontrol agents against important phytopathogens. *A. piperis* A/5 from composted plum-brandy production stillage displayed significant antagonistic activity against the important plant pathogen *P. aphanidermatum,* causing between 40-90% inhibition. According to the achieved inhibition, the A/5 isolate was comparable to some known *P. aphanidermatum* antagonists, such as *Trichoderma viride* 1433, which caused 72% inhibition in the confrontation test and 50.8% inhibition in glasshouse conditions [43]. Arunachalam and Sharma [21] reported that *P. aphanidermatum* was efficiently inhibited by 12

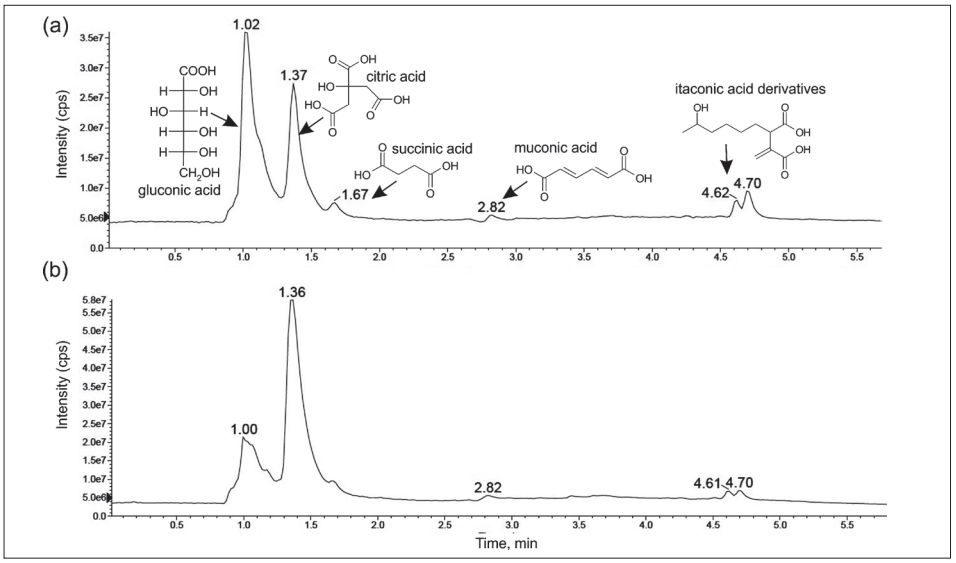

Fig. 4. LC-MS chromatograms (negative ion mode) and identification of small molecules in **a** – CCF and **b** – AP.

different isolates of *Trichoderma* sp. in the confrontation test in a range from 56.5% to 86.4%.

Aspergillus spp. that are members of section *Nigri* are important fungi in biotechnology, providing enzyme activities (amylase, lipase) and as organic acid (citric, gluconic) producers [33]. There are a few studies describing *Aspergillus* from section *Nigri* as antagonists to plant pathogens. In a similar study, 10 fungal isolates from compost were antagonistic to *F. oxysporum* f. sp. *melonis*, with the two exhibiting the highest biological control activity being Aspergillus spp., members of section *Nigri* [17]. *A. niger* was efficient in the suppression of rice sheath blight caused by *Rhizoctonia solani* [44].

To date, this is the first report of *A. piperis* as an antagonist of *P. aphanidermatum*. Some of the antagonists of *P. aphanidermatum* described so far include *Gliocladium virens*, *T. harzianum*, *T. viride*, *P. fluorescens*, *Agrobacterium radiobacter* K-84 and *Bacillus* sp. [7,43]. Biocontrol in terms of introducing a living organism to the environment has certain limitations associated with safety [45]. These limitations require particular caution, and research into some naturally occurred antagonistic interactions are often directed at the isolation and characterization of bioactive metabolites. Thus, we have examined the effect of the *A. piperis* A/5 CCF, its organic extract and the aqueous filtrate remaining after organic extraction (AP) on *P. aphanidermatum* growth.

We established that *A. piperis* A/5 was capable of producing a mixture of organic acids in the liquid medium. There are different findings about *P. aphanidermatum* tolerance to low pH in the environment. Pythium species are found in soils with a pH ranging from 3.6 to 7.2 [9]. The presented results suggest that the main potential of *A. piperis* for the biological control of *P. aphanidermatum* is not in the organic acid production.

We have also shown the antifungal activity as well as the presence of proteins and protein activities in the crude culture filtrate and in the aqueous phase remaining after solvent extraction (Table 2, Fig. 2 and Fig. 3). N-acetyl-β-glucosaminidase is related to chitin hydrolysis whereby it hydrolyzes N-acetyl- β-D-glucosamine residues from the terminal ends of chitooligosaccharides [46]. Purified β-glucosaminidase from *T. harzianum* demonstrated antifungal activity against several plant fungal pathogens, including *B. cinerea* [47]. It was purified and biochemically characterized as the 149 kDa protein from *A. niger* [48]. Furthermore, similar to our study,

N-acetyl-β-glucosaminidase isolated from *A. niger* 419 was characterized by some degree of thermostability and resistance to different organic solvents [49]. On the other hand, it was reported that fungal N-acetyl-β-glucosaminidase (72 kDa) [47] is larger than the proteins detected in this study. Considering that no N-acetyl-β-glucosaminidase activity was detected in the AP (Table 2), the enhanced activity of the AP was most likely due to some other proteinaceous compound with a lower molecular weight.

Peptides and small proteins exhibiting antimicrobial activity have been described as a promising tool for the development of new antifungal therapies [50, 51]. They have been shown to be effective in plant disease control as well [52]. Antifungal peptides derived from fungi are more active than those isolated from bacteria and plants [53]. Besides their activity, a significant advantage of antifungal peptides derived from filamentous fungi is their stability in various environmental conditions, such as pH, temperature, enzyme treatment [54]. Some of the isolated antifungal proteins are PAF from *Penicillium chrysogenum*, AnAFP from *A. niger*, AFP from *A. giganteus* and AcAFP from *A. clavatus* [38, 41, 55, 56]. However, they do not usually show antibacterial properties [41].

Taking into account our bioactivity results, it can be concluded that the presence of organic acids and derivatives inhibited proteins with antifungal activity. Microbial interactions within a complex compost community are important during the composting process and also after compost addition to soil [57]. Compost used in agriculture becomes part of the food chain and it is very important to prevent its contamination with human pathogens [58]. Plant disease suppression with compost is related to the microbial diversity that develops during compost maturation [18]. Therefore, the isolated *A. piperis* A/5 can be considered as a valuable multitasking compost community member, acting as a plant pathogen antagonist and thereby contributing to compost suppressiveness. In addition to the clarification of its role in agroecosystems, the presented results also indicate its potential in biotechnology, particularly in the production of organic acids and antifungal compounds.

Acknowledgments: This work was financially supported by the Ministry of Education and Science, Republic of Serbia, Grants No. TR31080 and No. ON173048, and EU Commission project AREA, No. 316004.

Authors' contributions: Jelena Jovičić-Petrović and Aleksandra Bulajić isolated and characterized fungal strains, Sanja Jeremić carried out bioinformatic analysis, Sandra Vojnović and Ivan Vučković characterized bioactive components of the fermentation broth. Vera Raičević and Jasmina Nikodinovic-Runic designed the study and prepared the manuscript.

Conflict of interest disclosure: The authors state that there is no conflict of interest.

REFERENCES

1. Perez-Garcia A, Romero D, de Vicente A. Plant protection and growth stimulation by microorganisms: biotechnological applications of Bacilli in agriculture. Curr Opin Biotechnol. 2011;22:187-93.
2. Pretty J, Bharucha ZP. Sustainable intensification in agricultural systems. Ann Bot. 2014;114(8):1571-96.
3. Hadar Y, Mandelbaum R, Gorodecki B. Biological control of plant diseases: progress and chaleges for the future. In: Tjamos EC, Papavizas GC, Cook RJ, editors. Proceedings of a NATO ARW held in Cape Sounion, Greece, May 19-24, 1991. New York: Plenum Press; 1992.
4. Jordan K, Dalmasso M, Zentek J, Mader A, Bruggeman G, Wallace J, et al. Microbes versus microbes: control of pathogens in the food chain. J Sci Food Agric. 2014;94:3079-89.
5. Bailey KL, Lazarovits G. Supressing soil-borne diseases with residue management and organic amendments. Soil Tillage Res. 2003;72:169-80.
6. Nzungize JR, Lyumugabe F, Busogoro J-P, Baudoin JP. *Pythium* root rot of common bean: biology and control methods: A review. Biotechnol Agron Soc Environ. 2012;16:405-13.
7. Jeyaseelan EC, Tharmila S, Niranjan K. Antagonistic activity of *Trichoderma* spp. and *Bacillus* spp. against *Pythium aphanidermatum* isolated from tomato damping off. Arch Appl Sci Res. 2012;4:1623-7.
8. Calvano TP, Blatz PJ, Vento TJ, Wickes BL, Sutton DA, Thompson EH, White CE, Renz EM, Hospenthal DR. *Pythium aphanidermatum* infection following combat trauma. J Clin Microbiol. 2011;49(10):3710-3.
9. Martin FN, Loper JE. Soilborne plant diseases caused by *Pythium* spp.: Ecology, epidemiology, and prospects for biological control. Crit Rev Plant Sci. 1999;18:111-81.
10. Haas D, Defago G. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol. 2005;3:307-19.
11. Perez-Piqueres A, Edel-Hermann V, Alabouvette C, Steinberg C. Response of soil microbial communities to compost amendments. Soil Biol Biochem. 2006;38:460-70.

12. Aranda V, Macci C, Peruzzi E, Masciandaro G. Biochemical activity and chemical-structural properties of soil organic matter after 17 years of amendments with olive-mill pomace co-compost. J Environ Manage. 2014;4797(14):00439-3.
13. Blaya J, Lloret E, Ros M, Pascual JA. Identification of predictor parameters to determine agro-industrial compost suppressiveness against *Fusariumoxysporum*and *Phytophthoracapsici*diseases in muskmelon and pepper seedlings. J Sci Food Agric. 2014;95(7):1482-90.
14. Riber L, Poulsen PH, Al-Soud WA, Skov Hansen LB, Bergmark L, Brejnrod A, Norman A, Hansen LH, Magid J, Sørensen SJ. Exploring the immediate and long-term impact on bacterial communities in soil amended with animal and urban organic waste fertilizers using pyrosequencing and screening for horizontal transfer of antibiotic resistance. FEMS Microbiol Ecol. 2014;90:206-24.
15. Bonanomi G, Antignani V, Capodilupo M, Scala F. Identifying the characteristics of organic soil amendments that suppress soilborne plant diseases. Soil Biol Biochem. 2010;42:136-44.
16. McKellar ME, Nelson EB. Compost-induced supression of *Pythium* damping-off mediated by fatty-acid metabolizing seed-colonizing microbial communities. Appl Environ Microbiol. 2003;69:452-60.
17. Suarez-Estrella F, Vargas-Garcia C, Lopez MJ, Capel C, Moreno J. Antagonistic activity of bacteria and fungi from horticultural compost against *Fusarium oxysporum* f. sp. *melonis*. Crop Prot. 2007;26:46-53.
18. Aviles M, Borrero C, Trillas MI. Review on compost as an inducer of disease suppression in plants grown in soilless culture. Dyn Soil Dyn Plant. 2011;5:1-11.
19. Kuter GA, Nelson EB, Hoitink HAJ, Madden LV. Fungal populations in container media amended with composted hardwood bark suppressive and conducive to *Rhizoctonia* damping-off. Phytopathol. 1983;73:1450-6.
20. Peper IL, Gerba CP, Brendencke JW. Environmental Microbiology. San Diego: Academic Press; 1995.
21. Arunachalam MK, Sharma P. Confrontation assay for *Trichoderma*as a potential biocontrol agent against *Pythium aphanidermatum*and *Sclerotinia sclerotiorum*. Pest Manag Hortic Ecosyst. 2012;18:74-7.
22. Cunnington JH, Takamatsu S, Lawrie AC, Pascoe IG. Molecular identification of anamorphic powdery mildews [Erysiphales]. Australas Plant Pathol. 2003;32:421-8.
23. He C, Fan Y, Liu G, Zhang H. Isolation and identification of a strain of *Aspergillus tubingensis* with deoxynivalenol biotransformation capability. Int J Mol Sci. 2008;9:2366-75.
24. Mule G, Susca A, Stea G, Moretti A. A species-specific PCR assay based on the calmodulin partial gene for identification of *Fusarium verticillioides*, *F. proliferatum* and *F. subglutinans*. Eur J Plant Pathol. 2004;110:495-502.
25. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997;25:3389-402.
26. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. Clustal W and Clustal X version 2.0. Bioinformatics. 2007;23:2947-8.
27. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol. 2013;30:2725-9.
28. Schwalbe R, Steele-Moore L, Goodwin A. Antimicrobial susceptibility testing protocols. New York: CRC Press; 2007.
29. Link AJ, LaBaer J. Trichloroacetic acid [TCA] precipitation of proteins. Cold Spring Harbor Protocols. 2011;8: pdb.prot5651.
30. Bradford M. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54.
31. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227(5259):680-5.
32. Perrone G, Stea G, Epifani F, Varga J, Frisvad JC, Samson RA. *Aspergillus niger* contains the cryptic phylogenetic species *A. awamori*. Fungal Biol. 2011;115:1138-50.
33. Varga J, Frisvald JC, Kocsube S, Brankovics B, Tóth B, Szigeti G, Samson RA. New and revisited species in *Aspergillus* section Nigri. Stud Mycol. 2011;69:1-17.
34. Samson RA, Houbraken JAMP, Kuijpers AFA, Frank JM, Fisvald JC. New ochratoxin A or sclerotium producing species in *Aspergillus* section Nigri. Stud Mycol. 2004;50:45-61.
35. Lopes FC, Tichota DM, Sauter IP, Meira SMM, Segalin J, Rott MB, Rios AO, Brandelli A. Active metabolites produced by *Penicilliumchrysogenum* IFL1 growing on agro-industrial residues. Ann Microbiol. 2013;63:771-8.
36. Abarca ML, Accensi F, Cano J, Cabanes F. Taxonomy and significance of black aspergilla. Antonie van Leeuwenhoek. 2004;86:33-49.
37. Parham JA, Deng SP. Detection, quantification and characterization of b-glucosaminidase activity in soil. Soil Biol Biochem. 2000;32:1183 -90.
38. Lee DG, Shin SY, Maeng CY, Jin ZZ, Kim KL, Ham KS. Isolation and characterization of a novel antifungal peptide from *Aspergillus niger*. Biochem Biophys Res Commun. 1999;263:646-51.
39. Lopez-Garcia B, Moreno AB, San Segundo B, De los Rios V, Manning JM, Gavilanes JG, Martínez-del-Pozo A. Production of the biotechnologically relevant AFP from *Aspergillus giganteus*in the yeast *Pichia pastoris*. Protein Expr Purif. 2010;70:206-10.
40. Skouri-Gargouri H, Ben Ali M, Gargouri A. Molecular cloning, structural analysis and modelling of the AcAFP antifungal peptide from *Aspergillus clavatus*. Peptides. 2009;30:1798-804.
41. Skouri-Gargouri H, Gargouri A. First isolation of a novel thermostable antifungal peptide secreted by *Aspergillus clavatus*. Peptides. 2008;29:1871-7.
42. Anita BS, Aree T, Kasettrathat C, Wiyakrutta S, Ekpa OD, Ekpe UJ, Mahidola C, Ruchirawata S, Kittakoop P. Itaconic acid derivatives and diketopiperazine from the marine-

derived fungus *Aspergillus aculeatus* CRI322-03. Phytochem. 2011;72:816-20.
43. Khare A, Singh BK, Upadhyay RS. Biological control of *Pythium aphanidermatum* causing damping-off of mustard by mutants of *Trichoderma viride*1433. J Agric Technol. 2010;6:231-43.
44. Kandhari J, Majumder S, Sen B. Impact of *Aspergillusniger* AN27 on growth promotion and sheath blight disease reduction in rice. Pest Sci Manag. 2000;2002:21-2.
45. Cook RJ. Assuring the safe use of microbial biocontrol agents: A need for policy based on real rather than perceived risks. Can J Plant Pathol. 1996;18:439-45.
46. Schomburg D, Salzmann M. N-Acetyl-beta-glucosaminidase. In: Schomburg D, Salzmann M, Gesellschaft für Biotechnologische Forschung (Braunschweig, Germany), editors. Enzyme Handbook 4. Berlin, Heidelberg: Springer; 1991. p. 195-201.
47. Lorito M, Hayes CK, Di Pietro A, Woo SL, Harman GE. Purification, characterization, and synergistic activity of a glucan 1,3-b-glucosidase and an *N*-acetyl-β-glucosaminidase from *Trichoderma harzanium*. Phytopathol. 1994;84:398-405.
48. Jones CS, Kosman DJ. Purification, properties, kinetics, and mechanism of beta-N-acetylglucosamidase from *Aspergillus niger*. Journal Biol Chem. 1980;255:11861-9.
49. Pera LM, InfanteMajolli MV, Baigori MD. Purification and characterization of a thermostable and highly specific beta-N-acetyl-D-glucosaminidase from *Aspergillus niger* 419. Biotechnol Appl Biochem. 1997;26:183-7.
50. Hancock REW, Sahl HG. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol. 2006;24:1551-7.
51. Meyer V. A small protein that fights fungi: AFP as a new promising antifungal agent of biotechnological value. Appl Microbiol Biotechnol. 2008;78:17-28.
52. Montesinos E. Antimicrobial peptides and plant disease control. FEMS Microbiol Lett. 2007;270:1-11.
53. Lucca AJ, Walsh TJ. Antifungal peptides: Origin, activity, and therapeutic potential. Rev Iberoam Micol. 2000;17.
54. Galgoczy L, Kovacs L, Vagvolgyi CS. Defensin-like antifungal proteins secreted by filamentous fungi. In: Mendez-Vilas A, editor. Current research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. Badajoz: Formatex Research Center; 2010. p. 550-9.
55. Marx F, Binder U, Leiter E, Pocsi I. The *Penicillium chrysogenum* antifungal protein PAF, a promising tool for the development of new antifungal therapies and fungal cell biology studies. Cell Mol Life Sci. 2008;65:445-54.
56. Nakaya K, Omata K, Okahashi I, Nakamura Y, Kolekenbrock H, Ulbrich N. Amino acid sequence and disulfide bridges of an antifungal protein isolated from *Aspergillusgiganteus*. Eur J Biochem. 1990;193:31-8.
57. Fuchs J. Interactions between beneficial and harmful microorganisms: From the composting process to compost application. In: Insam H, Franke-Whittle I, Goberna M, editors. Microbes at Work: From waste to resources. Heindbelrg: Springer-Verlag 2010. p. 213-30.
58. Gorny J. Microbial contamination of fresh fruits and vegetables. In: Sapers GM, Gorny J, Yousef AE, editors. Microbiology of fruits and vegetables. New York: CRC Press, Taylor and Francis Group; 2006.