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ANTIFUNGAL ACTIVITY OF MACROFUNGI EXTRACTS ON PHYTOPATHOGENIC FUNGAL STRAINS OF GENERA *Fusarium* sp. AND *Alternaria* sp.

ABSTRACT: During the last decades, intensive application of synthetic fungicides in the agricultural crop protection practice caused growing concern for the existence of toxic chemical residues in food as well as in the whole environment. Instead of using synthetic fungicides, it is suggested that crop protection be carried out by using preparations based on compounds of natural origin (secondary metabolites of plants or microorganisms, including macrofungi from Basidiomycota) as biological control agents. The potential of macrofungal species as biocontrol agents was analyzed in this investigation of eight autochthonous species from different locations in Serbia. Both the terricolous species: *Coprinus comatus*, *Coprinellus truncorum, Amanita strobiliformis, Hydnum repandum* and the lignicolous species: *Flammulina velutipes*, *Stereum subtomentosum*, *Trametes versicolor* and *Bjerkandera adusta* were examined, with an aim to detect some novel sources of antifungal agents. This study surveyed antifungal activity of selected macrofungal extracts (MeOH, EtOH and $CHCl_3$) against phytopathogenic *Fusarium* and *Alternaria* strains isolated from garlic, soybean and rice: *F. proliferatum*, *F. verticillioides*, *F. proliferatum*, *F. graminearum* and *A. padwickii*. Microdilution method in 96 well micro-plates was applied for the estimation of antifungal effects of macrofungi extracts in the range from 24.75 to 198.00 mg/ml and determination of minimal inhibitory (MIC) and minimal fungicidal concentration (MFC). EtOH extract of mychorhizal species *H. repandum* showed antifungal activity against all analyzed phytopathogenic strains, with the strongest effect on *Fusarium* strains (MIC 24.75 mg/ml; MFC 24.75 mg/ml). Among others, MeOH extracts of *S. subtomentosum* and *C. micaceus* showed similar effects while only *B. adusta* showed slight effect on *Fusarium* strains (MIC 24.75–99.00 mg/ml; MFC 24.75–99.00 mg/ml) and none effect on *A. padwickii*. The obtained results indicate the possibility of using examined extracts as efficient antifungal agents and provide the basis for the new formulations of biocontrol agents against phytopathogenic fungi in the future.

KEYWORDS: Biocontrol, antifungal activity, *Fusarium, Alternaria, Hydnum repandum*

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INTRODUCTION

Intensive application of synthetic fungicides in combat against phytopathogenic fungi has recently caused considerable concern, primarily due to toxic and carcinogenic chemical compounds found in food after fungicide application, as well as pollution caused by poor biodegradability of these compounds, and the development of pathogenic strains resistant to common commercial fungicides (Montesinos, 2003; Živković, 2016).

Interest in introducing alternative plant protection measures is therefore immense, especially concerning the research on novel organic fungicides, which are a form of biological control and an important natural phenomenon. Biological control is a way of protection of agro-ecosystems from harmful organisms, mainly pathogens, carried out by applying various agents and biopesticides such as microorganisms (bacteria, yeasts, macro-fungi) and their metabolic products or plants and plant extracts. Possibilities of applying macrofungal extracts for biological control of invasive organisms, especially phytopathogenic fungi, are therefore one of the top priorities in scientific research.

The term "macrofungi" relates to the fungi visible to the naked eye i.e. containing large fruiting bodies, which mainly belong to phyla Basidiomycota, Ascomycota (Chang and Miles, 2004). Macrofungi produce biologically active compounds, products of primary or secondary metabolism, with different activity: antifungal, antimicrobial, antioxidant, anti diabetogenic, anticarcinogenic, immunomodulatory (Kiho *et al*. 1996; Kim *et al*. 1999; Wasser, 2002; Lindequist *et al*. 2005; Karaman *et al*. 2009; Karaman *et al*. 2014). Macrofungi produce antimicrobial and antifungal metabolites so as to survive in their natural environment, thus the different macrofungal species (*Ganoderma carnosum, Hydnum repandum, Hygrophorus agathosmus, Lenzites betulina, Lepista nuda, Leucoagaricus pudicus, Paxillus involutus, Polyporus arcularius, Rhizopogon roseolus, Sarcodon imbricatus, Trametes versicolor*) can be used as a new source of natural compounds with antifungal activity (Yamaç and Bilgili, 2006).

Antifungal activity has been confirmed in 50 different species so far, including mostly the wild, edible macrofungi originating from Turkey, Portugal, China, Japan, Brasil, Hungary, Ireland and Malesia, namely *Flammulina velutipes, Hydnum repandum, Lentinus edodes, Ganoderma lucidum,* as well as the antifungal activity of various macrofungal extracts (Hirasawa *et al*. 1999; Hatvani, 2001; Smania *et al*. 2007; Hearst *et al*. 2009; Öztürk *et al*. 2011; Teoh *et al*. 2012; Alves *et al*. 2013; Heleno *et al*. 2013). Majority of the studies focus on antifungal activity towards one species of the human pathogen *Candida albicans* (Rosa *et al*. 2003; Kalyoncu i Oskay, 2008; Kalyoncu *et al*. 2010; Ozen *et al*. 2011), while others deal with phytopathogenic fungi *Fusarium verticillioides, Botrytis cinerea, Fusarium oxysporum, Physalospora piricola, Mycosphaerella arachidicola* (Wang, 2004; Lindequist *et al*. 2005; Wang, 2006; Gilardoni *et al*. 2007; Wang *et al*. 2012).

Since studies of autochthonous fungi of different geographical origin are important for discovering new isolates with prospective antifungal activity, the aim of the study was to draw attention to the use of "raw" extracts of the

selected macrofungi, such as *Coprinus comatus*, *Coprinellus truncorum, Amanita strobiliformis, Hydnum repandum, Flammulina velutipes*, *Stereum subtomentosum*, *Trametes versicolor*, and *Bjerkandera adusta,* in biocontrol of phytopathogenic fungi, namely *Fusarium* and *Alternaria* isolated from garlic, soybean and rice, as a prospective mode of plant protection.

MATERIAL AND METHODS

Preparing the suspension of filamentous phytopathogenic fungi

Isolation of five phytopathogenic fungi was carried out on a nutrient Potato Dextrose Agar agar (PDA) using the infested plant parts which showed symptoms of rot (Table 1). After growth and sporulation, fungi were isolated from the monospore cultures in order to obtain uniform isolates (Figure 1). Tested and confirmed, such obtained isolates are phytopathogenic (Ignjatov *et al*. 2016).

Table 1. Isolated phytopathogenic fungi and host plants*

Code	Pathogen	Hosts
BL1	*Fusarium proliferatum*	Garlic
BL4	*Fusarium verticillioides*	Garlic
BL5	*Fusarium proliferatum*	Garlic
S1	*Fusarium graminearum*	Soybean
ALT	*Alternaria padwickii*	Rice

* fungal collection – dr Maja Ignjatov

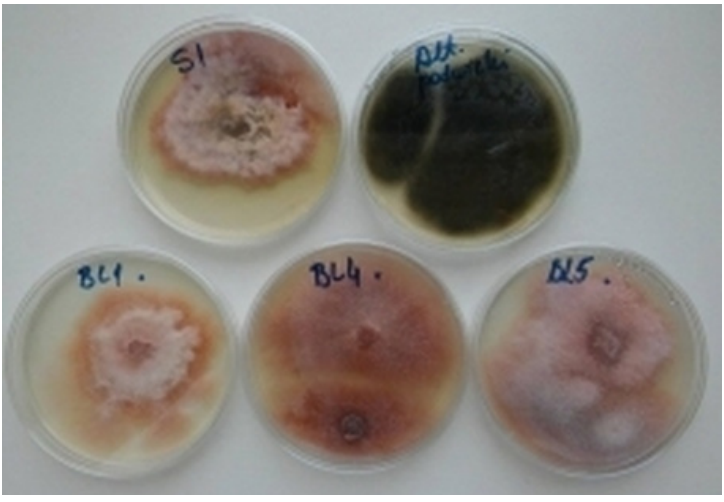

Figure 1. Analyzed phytopathogenic fungi: *F. graminearum* S1, *A. padwickii, F. proliferatum* BL1, *F. verticillioides* BL4 , *F. proliferatum* BL5 (collection – dr Maja Ignjatov)

Cultures were grown on PDA, which proved to promote fast growth and sporulation, within 7 days at 27 °C. After incubation under sterile conditions, a sample of the cultivated fungi was taken and suspended in sterile distilled water. Suspension of spores of *F. proliferatum* (BL1)*, F. verticillioides* (BL4), *F. proliferatum* (BL5) was at the density of 1.5 x 10^7 cells per ml (c/ml), while the observed density in *F. graminearum* (S1) and *A. padwickii* was 1.5 x 10^6 c/ml. Bürker Türk chamber (hemocytometer) and a microscope were used in order to obtain the adequate inoculum turbidity and the desired density. The number of spores in the chamber was determined by direct counting using a microscope (Olympus, BX51, Japan) in specific chamber cubes. The number of spores in a chamber is used to calculate the concentration or spore density in a suspension, using the formula:

$$\text{Mixture cell concentration} = \frac{\text{(no. of counted cells)}}{\text{(no. of chambers) x (chamber volume)}} \text{ x } 1000$$

Suspensions with adequate density were obtained (1.5 x 10^7 i 1.5 x 10^6 c/ml) using this formula.

Preparation of MeOH, EtOH, $CHCl_3$ macrofungal extracts

Eight autochthonous macrofungi were collected from different locations in Serbia (Fruska Gora, Tara and Sremski Karlovci), four of which were terricolous: *Coprinus comatus* (O.F. Müll.) Pers. 1797, *Coprinellus truncorum* (Scop.) Redhead, Vilgalys & Moncalvo 2001, *Amanita strobiliformis* (Paulet ex Vittad.) Bertill. 1866, *Hydnum repandum* L. 1753, and four lignicolous species: *Flammulina velutipes* (Curtis) Singer 1951, *Stereum subtomentosum* Pouzar 1964, *Trametes versicolor* (L.) Lloyd 1921, *Bjerkandera adusta* (Willd.) P. Karst. 1879.

Fungal fruiting bodies were first cleaned and lyophilized at -80 °C, under vacuum. The lyophilized mass obtained from the samples was measured (OHAUS explorer, ex 224M), and then extracted in the ratio of 1:10, using different solvents (70% MeOH, 80% EtOH, and 100% $CHCl_3$). Extraction was carried out in a mechanical mixture for 72 h (New Brunswick Scientific, Edison, USA) (100 rpm). Thereafter, the obtained extracts were filtered on a vacuum pump using filters paper Watman No. 1 (Fironi, Italy).

MeOH and EtOH extracts were then evaporated on a Rotavapor unit (Bűchi, R-210, Switzerland) at 50 °C, while the $CHCl_3$ extracts were evaporated at 40 °C. Thereafter, the leftover dried mass thus obtained was dissolved in a specific solvent so as to obtain the final concentrations of the extracts, i.e. 10%, 20% and 40% (w/w).

The following concentrations of extracts were obtained: 10% MeOH (*T. versicolor*), 20% MeOH (*F. velutipes*, *S. subtomentosum*, *H. repandum*, *B. adusta*), 40% MeOH (*A. strobiliformis, C. comatus*, *C. truncorum*), 20% EtOH (*H. repandum*, *A. strobiliformis*, *C. comatus*, *C. truncorum*), and 20% $CHCl_3$

(*C. comatus*, *C. truncorum*). The prepared extracts were kept in a refrigerator at +4 °C, until the next use.

Testing antifungal activity of MeOH, EtOH, $CHCl_3$ macrofungal extracts using the microdilution method

Antifungal activity of analyzed macrofungi was determined *in vitro* by microdilution method in 96 well microtiter plates (Spektar, Čačak, Serbia), so as to determine the minimal inhibitory and fungicide concentrations (MIC and MFC). Sterile polypropylene microtiter plates were used for this purpose . The total volume of a well was 101 µl. The amount of 50 µl nutrient broth (Malt broth, Torlak, Serbia) was applied into each well and 1 µl of phytopathogenic fungal spore suspension in 3 different double solutions (100%, 50% and 25%).

Microtiter plates were incubated for 72 h at 27 °C, and the results were read visually. The first concentration of extract without visible growth was taken as minimal inhibitory concentration (MIC), while minimal fungicide concentration (MFC) was determined after reading the MIC values, by transferring the whole volume of a well onto petri Malt Agar plates (Torlak, Serbia). The total volume was transferred to the Malt agar plates without any turbidity noticed. After incubation for 72 h at 27 °C, the results previously monitored by counting colonies were read.

RESULTS AND DISCUSSION

The results obtained by the microdilution method were presented (Table 2) through a parallel review of MICs and MFCs of macrofungal extracts towards phytopathogenic fungal strains: four *Fusarium* (*F. proliferatum* – BL1, *F. verticillioides* – BL4, *F. proliferatum* – BL5, *F. graminearum* – S1) and one *Alternaria* (*A. padwickii* – ALT).

Among the tested macrofungal extracts, antifungal activity to all the tested phytopathogenic fungi was displayed by the EtOH extracts of *H. repandum*, the strongest to strains *Fusarium* sp. (MIC 24.75 mg/ml; MFC 24.75 mg/ml), while somewhat lower to *A. padwickii* (MIC 24.75 mg/ml; MFC 49.50 mg/ml). On the other hand, the MeOH extract of the same species exhibited activity to *A. padwickii* (MIC 24.75 mg/ml), as well.

Besides, the EtOH extract of *H. repandum*, antifungal activity to all phytopathogenic isolates was exhibited by MeOH extracts of *S. subtomentosum* (MIC 49.50–99.00 mg/ml; MFC 49.50–99.00 mg/ml) and *C. truncorum* (MIC 99.00–198.00; MFC 99.00–198.00 mg/ml). MeOH extract of *B. adusta* had effect only on *Fusarium* (MIC 24.75–99.00; MFC 24.75–99.00 mg/ml), while the MeOH extracts of *C. comatus*, *T. versicolor*, *F. velutipes*, as well as EtOH extracts of *C. comatus*, *A. strobiliformis*, and the $CHCl_3$ extract of *C. comatus* had antifungal effects on the phytopathogenic isolate *A. padwickii*.

Table 2. MIC and MFC values (mg/ml) analyzed for MeOH, EtOH, and $CHCl_3$ macrofungal extracts towards phytopathogenic fungi (moulds)

Moulds→	*Fusarium proliferatum* (BL1)		*Fusarium verticillioides* (BL4)		*Fusarium proliferatum* (BL5)		*Fusarium graminearum* (S1)		*Alternaria padwickii* (ALT)	
Fungal extracts ↓	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
	concentration (mg/ml)									
	MeOH									
CcMeOH	nd	nd	nd	nd	nd	nd	nd	nd	99.00	nd
CtMeOH	198.00	198.00	198.00	198.00	198.00	198.00	198.00	198.00	99.00	99.00
AsMeOH	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
BaMeOH	99.00	99.00	49.95	49.95	49.95	49.95	24.75	24.75	nd	nd
HrMeOH	nd	nd	nd	nd	nd	nd	nd	nd	24.75	nd
TvMeOH	nd	nd	nd	nd	nd	nd	nd	nd	49.50	nd
FvMeOH	nd	nd	nd	nd	nd	nd	nd	nd	99.00	nd
SsMeOH	49.50	49.50	99.00	99.00	99.00	99.00	49.50	49.50	99.00	99.00
	EtOH									
CcEtOH	nd	nd	nd	nd	nd	nd	nd	nd	99.00	nd
CtEtOH	nd	nd	nd	nd	nd	nd	nd	nd	99.00	nd
AsEtOH	nd	nd	nd	nd	nd	nd	nd	nd	99.00	nd
HrEtOH	24.75	24.75	24.75	24.75	24.75	24.75	24.75	24.75	24.75	49.50
	$CHCl_3$									
$CcCHCl_3$	nd	nd	nd	nd	nd	nd	nd	nd	24.75	nd
$CtCHCl_3$	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

*nd – not detected, CcMeOH – MeOH extract *C. comatus*, CtMeOH – MeOH extract *C. truncorum*, AsMeOH- MeOH extract *A. strobiliformis*, BaMeOH – MeOH extract *B adusta*, HrMeOH – MeOH extract *H. repandum*, TvMeOH – MeOH extract *T. versicolor*, FvMeOH – MeOH extract *F. velutipes*, SsMeOH – MeOH extract *S. subtomentosum*, CcEtOH – EtOH extract *C. comatus*, CtEtOH – EtOH extract C. *trucorum*, AsEtOH – EtOH extract *A. strobiliformis*, HrEtOH- EtOH extract *H. repandum*, $CcCHCl_3$ – $CHCl_3$ extract *C. comatus*, $CtCHCl_3$ – $CHCl_3$ extract *C. trucorum*

Only in case of the MeOH extract of *A. strobiliformis* and $CHCl_3$ extract of *C. micaceus*, no antifungal effects on any of the tested phytopathogenic isolates were observed.

According to Aqueveque *et al.* (2016), MeOH extract of two species of the *Stereum* genus showed weak antifungal activity, exhibiting very weak antifungal activity to *B. cinerea* in only 7 out of 36 strains tested. In our study, MeOH extract of *S. subtomentosum* had a strong antifungal effect on all the tested phytopathogenic strains reaching MIC at 49.50–99.00 mg/ml and MFC at 49.50–99.00 mg/ml.

MeOH extracts of the both cultivated and wild *C. comatus* in the study of Stojković *et al.* (2013) exhibited strong antifungal effect on *Trichoderma viride* (MIC 0.25–1.50 mg/ml and MFC 1.50–3 mg/ml) and *Aspergillus versicolor* (MIC 0.20–0.75 mg/ml and MFC 1.50–3 mg/ml). Slightly weaker antifungal

activity to *Aspergillus fumgiatus* and *Penicllium verrucosum* was observed, while the lowest was detected for the MeOH extract of wild *Aspergillus niger*. In the present study, the MeOH extract of *C. comatus* exhibited no antifungal activity on *Fusarium*, whereas it was detected on *Alternaria* strain tested (MIC 99.00 mg/ml). According to Ehssan and Saadabi (2012), EtOH extract of *C. comatus* from Soudan did not exhibit antifungal activity on phytopathogenic strain *A. niger.* The result is partialy in accordance with our results obtained in the study which showed generally low antifungal activity of the EtOH extract of *C. comatus* (Table 2). EtOH extract of *Hydnum repandum* in the study of Yamaç *et al*. (2006) exhibited low antibacterial activity, (eight bacterial strains without activity and only one with < 10 mm) using the disc diffusion method, whereas high antifungal activity was observed in our study on *Fusarium* strains (MIC 24.75 mg/ml; MFC 24.75 mg/ml).

According to Alves *et al.* (2012) proteins and polysaccharides (β-Glucans) isolated from mushrooms showed antifungal activity. Earlier studies indicate that the diterpenoids and sesquiterpenoids from Basidiomycetes macrofungi showed antifungal activity against some phytopathogenic fungal strains: *Fusarium culmorum, Alternaria solani, Botrytis cinerea, Trichoderma lignorum* (Florianowicz, 1999; Liu, 2007). According to Wang *et al* (2012) sesquiterpenes, enokipodin F, G and I isolated from *F. velutipes* mycelium presented low activity against *Aspergillus fumigatus* with IC_{50} values 229.1 ± 3.6, 233.4 ± 3.8, 235.1 ± 4.2 μM respectively and the result is in accordance with our study which showed generally low antifungal activity of the MeOH extract of *F. velutipes* on all phytopathogenic strains.

CONCLUSION

Antifungal activity in all the tested phytopathogenic fungi was exhibited by the EtOH extract of *H. repandum,* with the strongest activity to *Fusarium* strains (MIC 24.75 mg/ml; MFC 24.75 mg/ml) and weaker activity to *A. padwickii* (MIC 24.75 mg/ml; MFC 49.50 mg/ml). Antifungal activity to all tested phytopathogenic fungi was found for MeOH extracts of *S. subtomentosum* and *C. truncorum*, but in higher concentrations (MIC 49.50–99.00 mg/ml; MFC 49.50–99.00 mg/ml and MIC 99.00–198.00; MFC 99.00–198.00 mg/ml, respectively). MeOH extract of the macrofungi *B. adusta* exhibited high antifungal activity to *Fusarium* (MIC 24.75–99.00 and MFC 24.75–99.00 mg/ml), while no activity to *A. padwickii* was observed. In extracts not exhibiting activity to *Fusarium* phytopathogenic fungi, minimal inhibitory concentrations to *A. padwickii* were detected. MIC of 24.75 mg/ml was detected in the MeOH extract of *H. repandum* and the $CHCl_3$ extract of *C. truncorum*, at the concentration of 49.50 mg/ml in metanol extract of *T. versicolor*, 99.00 mg/ml in MeOH and EtOH extract of *C. comatus*, EtOH extract of *C. truncorum*, MeOH extract of *F. velutipes*, and EtOH extract of *A. strobiliformis*.

Based on the results obtained in this study, we can conclude that some macrofungal extracts such as EtOH of *H. repandum*, MeOH of *S. subtomentosum*,

MeOH of *C. truncorum* and MeOH of *B. adusta* are potentially efficient antifungal agents, and can therefore be the basis of the formulations for preparations used in biocontrol against phytopathogenic fungi.

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АНТИФУНГАЛНА АКТИВНОСТ ЕКСТРАКАТА МАКРОГЉИВА НА ФИТОПАТОГЕНЕ СОЈЕВЕ ГЉИВА РОДОВА *Fusarium* sp. И *Alternaria* sp.

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РЕЗИМЕ: Током последњих деценија интезивна примена синтетичких фунгицида у заштити пољопривредних усева изазива све већу забринутост људи због присуства токсичних хемијских остатака у прехрамбеним производима као и целој околини. Уместо коришћења синтетичких фунгицида, препоручује се да се заштита усева врши коришћењем препарата на бази једињења природног порекла (секундарни метаболити биљака или микроорганизама укључујући и макрогљиве из раздела Basidiomycota) као агенсе биолошке контроле. У овом раду истраживан је потенцијал агенаса биолошке контроле за осам аутохтоних врста макрогљива с различитих локалитета у Србији. Обе, териколне врсте: *Coprinus comatus*, *Coprinellus truncorum, Amanita strobiliformis, Hydnum repandum* и лигниколне врсте: *Flammulina velutipes*, *Stereum subtomentosum*, *Trametes versicolor* и *Bjerkandera adusta* су истражене с циљем да се открију неки нови извори антифунгалних агенаса. У оквиру овог рада истражена је антифунгална активност одабраних екстраката макрогљива (метанолни, етанолни и хлороформски) против фитопатогених сојева *Fusarium* и *Alternaria* изолованих с белог лука, соје и пиринча: *F. proliferatum*, *F. verticillioides*, *F. proliferatum*, *F. graminearum* и *A. padwickii*. За процену антифунгалног ефекта екстраката макрогљива употребљена је микродилуциона метода микротитар плочама с 96 велова у опсегу концентрација од 24,75 до 198,00 mg/ml и детерминацију минималне инхибиторне (MIC) и минималне фунгицидне концентрације (MFC). Етанолни екстракт микоризне врсте *H. repandum* показао је атифунгалну активност према свим анализираним фитопатогеним сојевима, са најјачим ефектом према сојевима *Fusarium* (MIC 24,75 mg/ml; MFC 24,75 mg/ml). Између осталог, сличан ефекат показали су и метанолни екстракти *S. subtomentosum* и *C. micaceus,* док је само *B. adusta* имала благи ефекат на сојеве *Fusarium* (MIC 24,75–99,00 mg/ml; MFC 24,75–99,00 mg/ml), али не и на *A. padwickii*. Добијени резултати указују на могућност коришћења испитаних екстраката као веома ефикасних антифунгалних агенаса и самим тим они представљају основу за нове формулације биоконтролних агенаса против фитопатогених гљива у будућности.

КЉУЧНЕ РЕЧИ: биоконтрола, антифунгална активност, *Fusarium, Alternaria, Hydnum repandum*