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Full Length Research Paper

Bio-activities of extracts from some axenically farmed and naturally grown bryophytes

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The antifungal activity of dimethyl sulfoxide (DMSO) extracts of three bryophyte species, two mosses (*Atrichum undulatum* (Hedw.) P. Beauv., *Physcomitrella patens* (Hedw.) Bruch and Schimp) and a liverwort (*Marchantia polymorpha* L. ssp. *ruderalis* Bischl. and Boisselier), grown in nature and in axenic culture was evaluated by microdilution method against five fungal species (*Aspergillus versicolor, Aspergillus fumigatus, Penicillium funiculosum, Penicillium ochrochloron* and *Trichoderma viride*). All the investigated bryophyte extracts have been proved to be active against all fungi tested. In general, extracts made from material grown in the laboratory (*in vitro*) conditions express better anti-fungal activity comparing to those made from material grown in the nature. Some of the fungi tested react similarly to both extracts.

Key words: *Atrichum undulatum, Marchantia polymorpha, Physcomitrella patens*, bryophytes, antifungal activity.

INTRODUCTION

The investigation on various plant biological activities has reach lately great scientific interests since plant extracts are seen as a potential bio-farmaceuticals but also could be used in agriculture (Gupta et al., 2010; Sumathi and Parvathi, 2010; Solomon et al, 2010; Karatas and Ertekin, 2010). In such studies, the vascular plants receive highly attention while some other group of organisms like bryophytes remains neglected. They are recognized as the basal or first diverging lineage of land plants (Forrest et al., 2006). They are morphologically and biochemically diverse.

Bryophytes (that is, liverworts, hornworts and mosses) expressed interesting bioactivities (Dulger at al., 2005; Chobot et al., 2006; Sabovljevic et al., 2006, 2010; Milar et al., 2007; Singh et al., 2007; Tonguc and Mercili, 2007; Veljic et al., 2009, 2010; Ulka and Karadge, 2010). They are known to posses fungi as endobionts, as well as to develop mycorrhiza. However, relationships of bryophytes and fungi remain under-investigated. They are not common in the diet of other organisms and even more, most of the consumers avoid them. Besides anti-feeding effect, bryophytes are known to posses various relationships with microorganisms (protozoas, fungi, bacterias, algae) (Ando and Matsuo, 1984; Castaldo-Cobianchi et al., 1988; Asakawa, 1990; Basile et al., 1998; Sabovljevic et al., 2001) and contains a set of various known and unknown secondary metabolites (Xie and Lou, 2009).

Bryophytes, as a diverse group, are chemically still incompletely known although, many new compounds for science were described from them, mainly from liverworts (Sabovljevvic and Sabovljevic, 2008). They have rather rare use in the ethno-medicine comparing to vascular plants and rather few use is known in some traditional medicine. The reports on biological activities of bryophyte extracts and neglected and unknown potentials of these

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Abbreviation: DMSO (dimethyl sulfoxide).

Figure 1. Axenic *in vitro* culture of *M. polymorpha* ssp*. ruderalis.*

second biggest groups of land plants with ca. 25,000 species and much more infra-taxa worldwide are reviewed by Sabovljevic and Sabovljevic (2008, 2010). New and interesting compounds found in bryophytes emphasised them as new sources of agents, some of which can be extremely active e.g. Pejin et al. (2011) *in press* a, b. The huger bryophyte biomass productions are needed for any kind of massive uses and investigations. However, the problems of establishing *in vitro* and axenic cultures remains to deal with (Sabovljevic et al., 2003; Vujicic et al., 2009; Sabovljevic et al., 2010).

There are evidence that some bryophyte extracts can be used against fungi and to treat commercially serious plant infections like *Phytophtora infestans, Botrytis cinerea* and *Alternaria solani* (Frahm and Kirchhoff, 2002; Mekuria et al., 2005). So, there is huge undiscovered potential of bryophyte extract use as bio-pesticides, as well. The aim of this study was to compare bio-activities against fungi of three randomly selected bryophyte species (one liverwort and two mosses) and to estimate if there is a difference among material grown in the wildness and axenically grown plants in laboratory controlled *in vitro* conditions.

The moss *Atrichum undulatum* (*Polytrichaceae*) is widespread temperate species usually growing in forest flour. The tallous liverwort *Marchantia polymorpha* ssp. *ruderalis* (*Marchantiaceae*) appears at the edges of the rivers and rivulets but also in wet and shaded urban and suburban sites. The ephemeral moss *Physcomitrella patens* (*Funariaceae*) with short and quick life span appears in the spring time on the wet soils.

Plant material for biological activity studies (shoots/apical thalli parts) were collected either from native habitats (Belgrade and surrounding) in spring 2009 or it was obtained from *in vitro* culture, so that way axenically farmed, disposed of co-habiting organisms.

Axenically cultures (Figures 1, 2 and 3) were established from spores (*A. undulatum* and *P. patens*) and from gemmae (*M. polymorpha* ssp. *ruderalis*) as described in Sabovljević et al. (2009) and elaborated in Bijelovic et al. (2004), Sabovljevic et al. (2005, 2006) and Vujicic et al. (2010).

Uses of studied bryophytes in traditional medicine

The plants from the family *Marchantiaceae* are well-known traditional Chinese medicinal herbs extensively used to treat skin tumefaction, to protect the liver and to treat hepatitis, being also used as antipyretics (Chobot et al., 2006; Harris, 2008). Large numbers of marchantiaceaen plants occur in Chinese Guangxi Zhuang autonomous district such as *Marchantia polymorpha*, *Marchantia convoluta* and *Marchantia paleacea* and are used by local people. These species grow together and are difficult to distinguish one from another because of their morphological similarity. Besides, many studies on the chemical constituents and

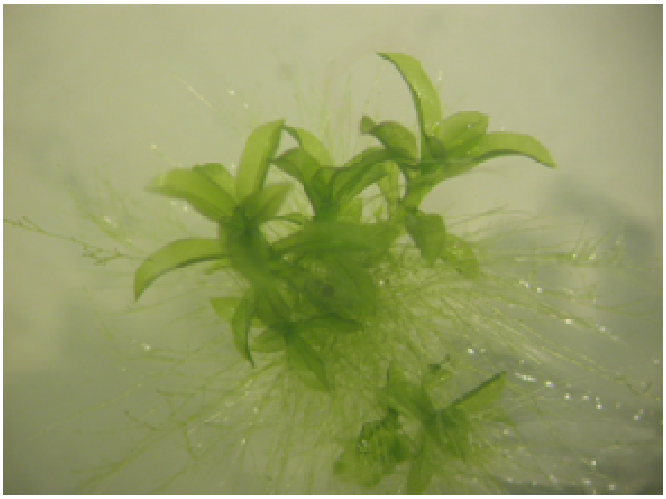

Figure 2. Axenic *in vitro* culture of *A. undulatum.*

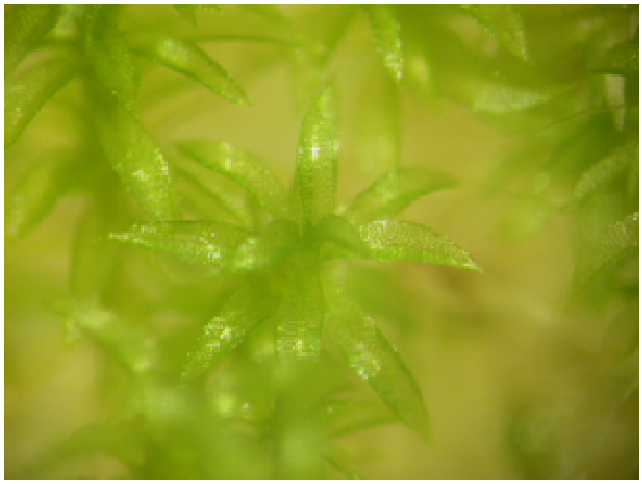

Figure 3. Axenic *in vitro* culture of *P. patens.*

bioactivities of *M. polymorpha s. lato* have been reported (Markham and Porter, 1974; Matsuo et al., 1985; Asakawa et al., 1987, 1990; Adam and Becker, 1994; Rieck et al., 1997; Asakawa, 2001; Neelam and Padma, 2008). The liverwort *M. polymorpha* has been used as a diuretic in traditional medicine for hundreds of years. Its pharmaceutical potency, however, was only recognized during the last three decades. Phytochemical investigations showed that besides terpenoids and flavonoids, bibenzyls and cyclic bis (bibenzyls) are also present in this plant. The most prominent examples of the group of bibenzyls are lunularic acid (Pryce, 1971), its decarboxylation product lunularine (Pryce, 1972) and the "prearomatic" precursor prelunularic acid (Ochta et al., 1983).

The labels with an *Atrichum* is reported to be seen on Chinese medicines primarily as anti-bacterial and anti-inflammatory agents (Glime, 2007). McCleary and Walkington (1966) considered that non-ionized organic acids and polyphenolic compounds might contribute to the antibiotic properties of bryophytes and found among eighteen mosses also *Atrichum* to inhibited one or both of Gram-positive and Gram-negative bacteria growth. *A. undulatum* extract was effective on everything tested except bacteria *Aerobacter aerogenes* and *Escherichia coli* (McCleary and Walkington, 1966). *Physcomitrella patens* is not known to posses traditional bioactivities.

Previously isolated classes of constituents in studied mosses

The liverwort *M. polymorpha* is known to posses many activities. However, until recently, under this name it was treated complex of species, it is not clear weather there is chemical distinction as well. Thus, it is well known by

marclocyclic bis(bibenzyls) – various types of marchantin, some of which besides antimicrobial are known to have anti-cancer effect (Asakawa et al., 1987, 2008; Chong et al., 2006).

The antibiotically active substances of *Atrichum* are considered to be polyphenolic compounds (McCleary and Walkington, 1966; Basile et al., 1999). It is also rich in flavonoids (Zinsmeister and Mues, 1980; Basile et al., 1999). Glycosides of three- and tetraoxygenated coumarins and polyhydroxilated daphin coumarin were reported from *A. undulatum* (Jung et al., 1994; Chobot et al., 2008). Chobot et al. (2008) also reported relatively strong antioxidant activity. Although, *P. patens* is bryophyte model system and has completely sequenced genome, the chemical constituent of this moss is not known, except for protein content (Skripnikov et al., 2009).

MATERIALS AND METHODS

Plant material and extract preparation

A. undulatum and *M. polymorpha* gametophytes were grown on MS medium (Murashige and Skoog, 1962) enriched with 0.1 M sucrose, while *P. patens* gametophytes were grown on BCD medium enriched with 0.1 M sucrose (Sabovljević et al., 2009). In order to investigate how the environmental conditions influence bryophyte relationships with other species, they were grown either on solid or liquid MS/BCD medium, as indicated in the Table 1.

The pH of the growth media was adjusted to 5.8 prior to autoclaving at 114°C for 25 min. Cultures were grown at 25 ± 2°C under the long day conditions (16/8 h of light to darkness) or under dark condition (24 h of dark), as indicated in the Table 1. Light was supplied by cool-white fluorescent tubes at a photon fluency rate of 47 µmol/m. Cultures were subcultured for a period of 4-6 weeks. Voucher specimens of *A. undulatum, M. polymorpha* ssp. *ruderalis* and *P. patens* have been deposited in the BEOU bryophyte collection (No. 4463, 4556, 4509).

The dimethyl sulfoxyde (DMSO) extracts of the studied bryophyte species were made from the specimens collected in the nature and their counterparts axenically grown in *in vitro* conditions. The DMSO extract were made in both cases only from the green shoots (mosses) and apical thallus parts (liverwort). DMSO was chosen because of its property to be inert and not to show any activity as compared with other dissolver like methanol or ethanol, for example.

The DMSO extract were made from shoots of *A. undulatum, M. polymorpha* ssp. *ruderalis* and *P. patens* grown in nature, on 0.1 M sucrose enriched MS or BCD medium, under the light or dark conditions (average yields per species: 8.9, 7.6 and 7.2%). All bryophyte samples (10 g) were dried by airflow at room temperature. They were then finely ground with a hammer mill and extracted separately with 1 ml of DMSO for 24 h at room temperature. Extracts were filtered with cellulose-acetate membrane (0.45 µm).

Test for antifungal activity

For the bioassays, five fungi were used: *Aspergillus versicolor* (ATCC 11730), *Aspergillus fumigatus* (ATCC 9142), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), *Trichoderma viride* (IAM 5061). All of the organisms tested were from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research «Siniša Stanković», Belgrade, Serbia. The micromycetes were maintained on malt agar (MA) and cultures were stored at +4°C and sub-cultured once a month (Booth, 1971).

Microdilution method

In order to investigate the antifungal activity of the extracts tested, the modified microdilution technique was used (Hanel and Raether, 1988; Daouk et al., 1995). The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 µl per well. The inocula were stored at 4°C for further use. Dilutions of the inocula were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculums. Minimum inhibitory concentrations (MICs) determination was performed by a serial dilution technique using 96-well microtitre plates. The extracts were added in broth medium with fungal inoculum to achieve required concentrations (0.5 to 10 mg/ml). The microplates were incubated for 5 days at 28°C. The lowest concentrations without visible growth (at the stereo microscope) were defined as concentrations which completely inhibited fungal growth (MICs).

The minimum fungicidal concentrations (MFCs) were determined by serial subcultivation of a 2 µl into microtitre plates containing 100 Ml of broth per well and further incubation for 5 days at 28°C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. DMSO was used as a control, while bifonazole and ketoconazole were used as positive control (0.1 to 2 mg/ml).

RESULTS AND DISCUSSION

The antifungal activities of tested extracts are reported in the Table 1. It was obvious that all the extracts showed better antifungal activity than antibacterial (compared to Sabovljević et al., 2010). MIC of extracts was ranged from 0.1 to 1.0 mg/ml and MFC is from 0.5 to 2.0 mg/ml. The best antifungal activity was obtained for extracts of *M. polymorpha* grown on MS liquid medium, *A. undulatum* grown on MS solid medium and *P. patens* grown on BCD solid medium with MIC of 0.1 to 0.5 mg/ml and MFC is 0.5 to 1 mg/ml. The lowest antifungal activity could be seen for all tested bryophyte extracts of selected species grown in nature, with MIC of 0.5 to 1.0 mg/ml and MFC of 1.0 to 2.0 mg/ml. The most sensitive micromycetes was *Trichoderma viride* while the most resistant species was *Penicillum ochrochloron.*

Bifonazole possessed inhibitory activity of 0.1 to 0.2 mg/ml and fungicidal of 0.2 to 0.25 mg/ml, while ketoconazole showed lower potential with MIC of 0.2 to 2.5 mg/ml and MFC of 0.5 to 3 mg/ml. It was obvious that extracts of all bryophyte species grown *in vitro*, on MS or BCD medium, showed better inhibitory activity than both known antifungal drugs against *T. viride.*

It could be seen that the growth of tested fungi responded differently to the compounds tested, which indicates that different components might have different

Table 1. Minimal inhibitory (MIC mg/ml; first row) and fungicidal concentration (MFC mg/ml; second row) of compounds tested.

Fungi	*M. polymorpha*				*A. undulatum*			*P. patens*		Bifonazole	Ketoconazol
	MS solid medium, light	MS liquid medium, light	MS solid medium, dark	Nature	MS solid medium, light	MS solid medium, dark	Nature	BCD solid medium, light	Nature		
T. viride	0.25	0.1	0.1	1.0	0.1	0.1	1.0	0.1	1.0	0.2	2.5
	0.5	0.5	0.5	1.0	0.5	0.5	1.0	0.5	1.0	0.25	3.0
P. funiculosum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.2	0.2
	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.25	0.5
P. ochrochloron	0.5	0.5	0.5	1.0	0.5	0.5	1.0	0.5	1.0	0.15	0.2
	1.0	1.0	1.0	2.0	1.0	1.0	2.0	1.0	2.0	0.2	0.5
A. fumigatus	0.25	0.5	0.5	0.5	0.5	1.0	0.5	0.5	0.5	0.15	0.2
	0.5	1.0	1.0	1.0	1.0	2.0	1.0	1.0	1.0	0.2	0.5
A. versicolor	0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.0	0.1	0.2
	0.5	1.0	1.0	1.0	1.0	1.0	2.0	1.0	2.0	0.2.	0.5

modes of action or that the metabolism of some fungi was able better to overcome the effect of the agents or adapt to it.

The results presented here, shown that some axenic culture were more effective to fungi tested, which can be explain by the better production of some of the effective extract constituents in artificial conditions rather than in nature. Further chemical studies are needed to confirm this hypothesis for this purpose; significantly higher biomass amount is needed.

Axenically grown bryophytes expressed to some extent the potential in biotechnological processes. Since a variety of relationships of tested bryophyte extracts with selected fungi were shown, the target species and the growth condition should be adjusted to achieve the maximum (parwise selection) when widely applied.

The problem how to achieve the huge amount of clean material remained and one of solution was to develop axenic cultures. The biomass production trough biotechnological processes not only gave enough material of these tiny plants, but as well prevent extinction and could be used in *ex situ* conservation (Rowntree et al., 2010). Thus, bryophyte axenic culture offer the possibility for achieving enough amounts of the agents that can be used even applied in nature for combating plant fungal infections in environmental friendly manner and not harmful to other living beings.

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