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The potential use of the culture filtrate of an *Aspergillus niger* strain in the management of fungal diseases of grapevine

Egy *Aspergillus niger* törzs fermentlevének lehetséges felhasználása a szőlő gombás betegségeinek megelőzésére

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ABSTRACT

The present study was aimed to examine the use of culture filtrates of defensin-producing fungal strains against the most important fungal and pseudofungal infections of grapevine as an environmentally sound alternative of chemical control. Twenty-seven isolates of five defensin-producing fungal species were tested for antifungal activity. Four isolates (one *Aspergillus giganteus* and three *Aspergillus niger*) decreased the viability of the test organism successfully. The isolate with the highest antifungal efficacy (*Aspergillus niger* SZMC2759) was subjected to detailed investigation. The culture filtrate of the strain was effective against epicuticular mycelia and conidia of *Botrytis cinerea*, *Guignardia bidwellii* and *Erysiphe necator*, but not against subcuticular mycelia and *Plasmopara viticola* cells. The culture filtrate also prevented the development of powdery mildew symptoms on grapevine cuttings. Several modes of action were detected: inhibition of germination, membrane permeabilization, plasmolysis, fragmentation of nuclei. Certain parameters (pH, stress, aeration) of the fermentation were optimized for the highest antifungal activity. According to the results, culture filtrate of *Aspergillus niger* can be a promising agent in the management of fungal diseases of grapevine.

Keywords: *Vitis vinifera*, fungal pathogens, disease control, antimicrobial peptide

ÖSSZEFOGLALÓ

Jelen tanulmány célja defenzint termelő gombafajok fermentleveinek a szőlő legfontosabb gombás betegségei ellen történő felhasználhatóságának vizsgálata, amely környezetbarát alternatívát nyújthat a vegyszeres növényvédelemmel szemben. Öt defenzint termelő gombafaj 27 izolátuma képezte a vizsgálatok tárgyát. Ezek közül négy (egy *Aspergillus giganteus* és három *Aspergillus niger*) izolátum volt képes hatékonyan csökkenteni a tesztorganizmus életképességét. A leghatékonyabb izolátumot (*Aspergillus niger* SZMC2759) részletes vizsgálatoknak vetettük alá. A törzs fermentleve fungicid hatást mutatott az epikutikuláris gombafonalakkal és spórákkal szemben a *Botrytis cinerea*, *Giugnardia bidwellii* és *Erysiphe necator* kórokozók esetén, de hatástalan volt a szubkutikuláris gombafonalak, valamint a *Plasmopara viticola* kórokozóval szemben. A vizsgált fermentlé képes volt tesztfertőzésekben a szőlő lisztharmat tüneteinek kialakulását

is meggátolni mesterségesen fertőzött dugványokon. A fermentlé számos gombaellenes hatásmechanizmusát sikerült igazolni: spórák csírázásának gátlása, plazmamembrán permeabilizálása, plazmolízis indukálása és sejtmag feldarabolódása. A fermentáció egyes körülményei (pH, stressz, oxigénellátottság) optimalizálásra kerültek a legnagyobb hatékonyság elérése érdekében. Eredményeink szerint az *Aspergillus niger* gombafaj fermentleve ígéretes gombaellenes szer lehet a szőlő növényvédelmében.

Kulcsszavak: *Vitis vinifera*, kórokozó gombák, növényvédelem, antimikrobiális peptidek

INTRODUCTION

The increasing demand of agricultural products for the growing global human population is a great challenge for agricultural companies. The widespread use of fertilizers and chemicals drastically increased the productivity of cultivation techniques in the last century (Popp et al., 2013). As a result of the climate change, the growing pressure of diseases on agriculture may increase the consumption of microbicidal sprays, especially in areas with mild climate (Pautasso et al., 2012). The ecological impact of these chemicals and the unpredictable effects of their accumulation in the food chain (Aktar et al., 2009) raised the demand of ecologically and medically safe alternatives. As a part of integrated pest and disease management, biological control has an increasing significance. For this purpose, living microorganisms (Harper, 2013) or microbial products (Stockwell and Duffy, 2012) are used to protect plantations from pests and pathogens.

Grape is one of the most spray-consuming agricultural product, primarily because of the fungal (e.g. black and grey rot, powdery mildew) and pseudofungal (downy mildew) diseases of grapevine. Black rot of grapes is caused by the ascomycetous fungus *Guignardia bidwellii*. This fungus is a hemibiotrophic pathogen, which grows as subcuticular mycelia after the penetration of germ tubes into the host epidermis. The pathogen causes significant problems mainly in North America, which may result in as high as 80% yield loss without management (Amsdell and Milholland, 1988). Grey rot of grape is caused by *Botrytis cinerea*, a necrotrophic fungal pathogen which also belongs to phylum Ascomycota. This fungus is a widespread disease agent which can infect more than 200 different crops (Williamson et al., 2007). Grey rot causes about 2 billion $US per year losses for winegrowers by reducing grape yields and quality (Elad et al., 2004). *Botrytis cinerea* can infect all parts of grapevines, but it mostly occurs on berries and colonizes the subcuticular tissues. Pseudofungal species *Plasmopara viticola*, the causative agent of downy mildew belongs to Oomycetes and it is an obligate biotrophic parasite of different species of the Vitaceae family. This pathogen grows mainly under the epidermis and only the reproductive structures form on the surface of the leaves. Downy mildew is a very severe disease of grapevines. With permissive weather conditions and without chemical control it can cause 70% yield losses (Gessler et al., 2011). Powdery mildew is caused by the ascomycetous fungus *Erysiphe necator*. This biotrophic pathogen can highly decrease grapevine productivity, and it negatively alters the quality of the affected musts (Calonnec et al., 2004). This fungus grows on the surface of the leaves and berries, only its haustoria, specialized structures for the plant-fungus interaction, are localized under the epidermis of the host. Several low-price and effective chemicals are used for the control of the above pathogenic organisms, but the ecological and medical risks are also concern to these agents. Moreover, the non-specific management of grape microflora may impair spontaneous fermentation of musts. This way of fermentation is preferred more than the use of commercial yeasts by some producers, because of the enhanced aroma complexity and the area-specific characteristics of the produced wine (Medina et al., 2013).

Defensins are antimicrobial peptides widely distributed in the entire eukaryotic living world. They can be found in fungi (Wu et al., 2014), plants (Stotz et al., 2009), insects (Hoffman and Hetru, 1992) and vertebrates (Ganz 2004), for example as a part of the

innate immune system (Oppenheim et al., 2003). These peptides are very effective against a wide range of pathogens including viruses, bacteria and fungi. They can act in several different ways, which decreases the risk of the development of resistance against them. Defensins possess low molecular weight, alkaline character and contain 6-10 cysteine residues, which form disulphide bonds (Sahl et al., 2005). The compact structure and the high number of disulphide bonds result in high structural and functional stability. Defensins can withstand immoderate pH and temperature conditions or proteolytic cleavage (Batta et al., 2009; Lacadena et al., 1995; Theis et al., 2005). These properties make them promising agents for both healthcare and agriculture. The fungal defensins are the most suitable for industrial production due to the simple handling of the producing organisms. Cytotoxicity experiments have shown that fungal defensins have no harmful effects on plant or animal cells (Meyer, 2008; Szappanos et al., 2006). The applicability of fungal defensins against plant pathogens have been tested in numerous studies (Coca et al., 2003; Girgi et al., 2006; Moreno et al., 2003; Moreno et al., 2005; Oldach et al., 2001; Vila et al., 2001) by using defensins as sprays or expressing these antimicrobials in plants. However, none of these studies focused on the applicability of these peptides against various fungal pathogens of the grapevine. The purpose of the investigation was to examine the potential use of some culture filtrates of defensin-producing fungal species for the biological control of fungal grapevine diseases.

MATERIALS AND METHODS

Strains and growth conditions

For antifungal activity tests, strains of defensin-producing fungal species were obtained from the Szeged Microbiology Collection, Department of Microbiology, University of Szeged (Szeged, Hungary, http://szmc.hu) and listed in Table 1. *Botrytis cinerea* and *G. bidwellii* strains were isolated from the vineyard of Eszterházy Károly University, Research Institute for Viticulture and Enology (Eger, Hungary) and identified according to morphological characteristics. All strains were maintained by cultivation on PDA medium (0.4 w/v% potato extract, 2 w/v% glucose, 1.5 w/v% bacteriological agar in distilled water) at 25 °C. The obligate biotrophic *E. necator* and *P. viticola* samples were collected freshly from *Vitis vinifera* cv. Kékfrankos plants without disease control.

Table 1. List of fungal strains used in screening for antifungal activity

Strain identifier	Species
SZMC0145	*Aspergillus niger*
SZMC0514	*Penicillium chrysogenum*
SZMC0918	*Aspergillus clavatus*
SZMC12629	*Penicillium chrysogenum*
SZMC12641	*Penicillium chrysogenum*
SZMC12669	*Penicillium chrysogenum*
SZMC12676	*Penicillium chrysogenum*
SZMC13619	*Rhizopus microsporus*
SZMC13622	*Rhizopus microsporus*
SZMC1389N	*Neosartorya fischeri*
SZMC2028A	*Aspergillus clavatus*
SZMC2042	*Aspergillus clavatus*
SZMC2061N	*Neosartorya fischeri*
SZMC2071	*Aspergillus clavatus*
SZMC20728	*Aspergillus clavatus*
SZMC2161	*Aspergillus niger*
SZMC2170	*Penicillium chrysogenum*
SZMC2176	*Aspergillus niger*
SZMC2182	*Aspergillus niger*
SZMC2192	*Aspergillus niger*
SZMC2197	*Aspergillus niger*
SZMC2207	*Aspergillus niger*
SZMC2356	*Neosartorya fischeri*
SZMC2402	*Aspergillus niger*
SZMC2660	*Aspergillus niger*
SZMC2719	*Aspergillus clavatus*
SZMC2759	*Aspergillus niger*

Preparation of fermentation broths

Fungi to be tested for antifungal activity were grown in liquid YG medium (0.5 w/v% yeast extract, 2 w/v% glucose in distilled water) under static conditions. All fermentations were run at 25 °C for 7 days. The fungal strain, selected for the highest antifungal activity, was cultivated under altered conditions. To examine the effect of the initial pH on the antifungal efficiency of the fermentation broth, medium was adjusted to pH 4, 5, 6, 7 and 8 with HCl and NaOH. For the investigation of the effects of stress factors, the medium was supplemented with 1 M NaCl (osmotic stress) or 0.3 v/v% Tween 80 (membrane stress). In order to examine the influence of aeration, fermentations with dynamic conditions (180 rpm stirring) were also carried out. All fermentation broths were filtered through a 0.2 µm pore size membrane to obtain a cell-free filtrate.

Germination ability tests

To test the effect of fungal culture filtrates on spore germination, conidial suspensions of *G. bidwelli* and *B. cinerea* (10^6 cell/ml) were prepared using 14-day old colonies cultivated on PDA medium. 50 µl aliquots of the suspensions were mixed with 50 µl of fungal culture filtrate or YG medium (control) and spread on the surface of cellophane placed on PDA medium. Conidia were incubated at 25 °C for 8 hours then examined by microscope. The percentage of germinating spores was calculated. The assays were performed in triplicates.

Viability tests

Thiazoyl blue tetrazolium bromide reduction assay

For the quantification of antifungal activity of fungal culture filtrates by thiazolyl blue tetrazolium bromide (MTT) reduction assay three agar plugs with young mycelia were cut by cork borer (3 mm in diameter) from the edge of 7 day old *G. bidwellii* colonies on PDA medium and incubated in 500 µl fungal culture filtrate or YG medium (control) for 12 h at 25 °C. Thereafter broths were discarded and replaced with 500 µl of 5 mM MTT (Sigma Aldrich) in distilled water. To allow the living cells to convert yellow MTT to purple formazan the samples were incubated for 3 h at 25 °C. The liquid phase was discarded and the samples were incubated with 1 ml extraction solution (0.04 M HCl in 2-propanol) for 2 h at 60 °C to dissolve the produced formazan. Formazan contents were determined through spectrophotometric measurement (SmartSpec Plus, Bio-Rad) at 575 nm wavelength. The viability of mycelial cells was expressed as percentage relative to the untreated control. The investigations were carried out in triplicates.

Fluorescein diacetate hydrolysis assay

For the quantification of biocidal activity of fungal culture filtrates by fluorescein diacetate (FDA) hydrolysis assay suspensions of conidia were prepared from *B. cinerea*, *E. necator*, *G. bidwellii*, while from *P. viticola* sporangial suspension was made. All suspensions were adjusted to 10^6/ml by sterile distilled water. A 100 µl aliquot of suspensions were mixed with the same volume of the serial dilutions of fungal culture filtrate and incubated for 4 h at 25 °C. Thereafter the mixtures were supplemented with 10 µM FDA (Sigma Aldrich) and incubated for additional 30 min. For calibration, the cell suspensions were serially diluted and treated as described previously, except that YG medium was used instead of culture filtrate. Fluorescence was detected and documented by Gel Doc XR System (Bio-Rad). Quantification of fluorescence was done by imageJ software (Schneider et al., 2012). The percental viability of treated cells was calculated by the aid of the calibration.

Characterization of the active fungicidal agent of culture filtrate

Fungicidal activity test of protein fraction of culture filtrate

The antifungal activity of the protein fraction of culture filtrate was tested against *G. bidwellii* conidia after precipitation by trichloroacetic acid (TCA). Briefly, 25 µl of 100 w/v% TCA solution was added to 100 µl of culture filtrate and incubated for 1 h on ice. Proteins were pelleted by centrifugation at 12000 g for 10 min and washed twice with 200 µl ice-cold acetone. The pellet was dried and

resuspended in 100 µl distilled water. Protein fractions were also prepared in reducing conditions. Reduced samples are obtained as described above, with a previous 2-mercaptoethanol (2-ME) treatment (incubation with 100 mM 2-ME for 5 h, at 30 °C) to examine the possible role of disulphide bonds in the fungicidal agent. The viability of treated and control conidia of *G. biwellii* was measured by fluorescein diacetate hydrolysis assay as described above. All experiments were conducted in triplicates.

Estimation of the molecular weight of fungicidal agent by gel-electrophoresis and bioautography

Bioautography tests were done by sodium dodecyl-sulphate acrylamide gel electrophoresis (SDS-PAGE) with non-reducing conditions and the subsequent detection of fungicide activity of culture filtrate. Culture filtrate was supplemented with 5 v/v% glycerol, 2 w/v% SDS, traces of bromophenol blue and 50 mM Tris (pH 8). Samples were incubated at room temperature for 30 min prior to electrophoresis. Electrophoresis was carried out in a discontinuous (5% T stacking and 15% T resolving gel) polyacrylamide gel (Laemmli, 1970), at 30 mA current at 4 °C until bromophenol blue reached the bottom of the gel. SDS was removed from the gel by washing twice with 20 v/v% 2-propanol for 20 min. The fungicide activity was detected by agar overlay method. Briefly, solid YG medium at 50 °C temperature was supplemented with 10^5/ml *Saccharomyces cerevisiae* cells (CGC 62, Uvaferm) and poured on top of the acrylamide gel. The gel was incubated at 30 °C for 8 hours after solidification. Viable cells were detected by staining the medium with 5 mM MTT at 30 °C for 3 hours.

Microscopic examinations

For the microscopic examination of the effects of culture filtrates on fungal cells, small sections of young mycelia were cut from the edge of 7-day old *G. bidwellii* or *B. cinerea* colonies growing on PDA medium. The mycelia were immersed in 100 µl of the tested culture filtrate or YG medium at 25 °C for 4 hours. Samples were stained with 0.4 w/v% Crystal Violet for 20 min and washed with distilled water to visualize cytoplasm. For the staining of nuclei mycelia were stained with 10 µg/ml SYBR GREEN I (Sigma Aldrich) for 15 min. For the assessment of cell membrane permeability mycelial samples were stained with 30 µg/ml ethidium bromide (EtBr) for 30 min then washed with distilled water. Viability stainings were done with asexual spores of *B. cinerea*, *E. necator*, *G. bidwellii*, *P. viticola* or leaf discs expressing the symptoms of *E. necator* or *G. bidwellii* infections. Samples were treated with culture filtrate or YG medium (control) at 25 °C for 4 hours. Staining was done by the use of FDA as described previously in FDA fluorescence assay. Leaf discs with *G. bidwellii* infections were treated with 1 unit/ml *A. niger* pectinase (Fluka) in citrate-phosphate buffer (pH 4) at 25 °C for 2 hours prior to staining. All examinations were carried out by Alpha BIO-5F microscope (Alpha). Images were taken by the use of Artcam-500MI camera (Artray) and Quickphoto Camera 2.3 software (PROMICRA).

Testing of antifungal activity of culture filtrate on grapevines cuttings

Examination of in planta antifungal effect of culture filtrates was done by the use of one-year old *V. vinifera* cv. Kékfrankos cuttings. Cuttings were obtained by planting two-bud cane sections in soil:perlite (1:1) mixtures placed in plastic pots. Plants were grown in a greenhouse under natural light cycle, without artificial lighting and humidity control. The air temperature of the greenhouse was half-controlled by an automatic system, which regulated the opening of the upper windows during summer. Leaves were inoculated by spraying conidial suspension of E. necator (10^5 conidia/ml, prepared in distilled water) on the leaves. Artificially infected cuttings were incubated in the greenhouse under the conditions described above, except that the plants were covered with plastic bags for the first three days of the experiment (27.-29. of July, 2016) to provide increased humidity. After one day, the grapevines were sprayed with YG medium (control) or fungal culture filtrate. Plants were incubated for an additional four weeks, and the spray treatments were repeated weekly. Symptoms of powdery mildew were examined visually. All experiments were done in triplicates.

Statistical analysis

Statistical analysis was done by two-way ANOVA analysis using GraphPad Prism 5 software demo version (GraphPad Software, San Diego California USA, www.graphpad.com). Diagrams were generated with the same software and the layout was edited by Adobe Photoshop CS5 demo version.

RESULTS

Screening of fungal culture filtrates for antifungal activity

Culture filtrates of 27 isolates from five species were tested for antifungal activity against *G. bidwellii* mycelia by MTT assay (Figure 1). One *A. clavatus* (SZMC20728) and 3 *A. niger* (SZMC0145, SZMC2176, SZMC2759) isolates showed high antifungal activity (more than 50% loss of viability). Repeated experiments with dilutions of culture filtrates showed that the culture filtrate of SZMC2759 (AnCF) had the highest antifungal potential. Further works were focused on the study of this strain.

Determination of fungal and pseudofungal pathogens of grapevine susceptible to AnCF

The biocidal activity of AnCF was tested by FDA assay against the asexual spores of the most important fungal and pseudofungal pathogens of grapevine: *B. cinerea* (grey rot), *E. necator* (powdery mildew), *G. bidwellii* (black rot) and *P. viticola* (downy mildew). The results are presented in Figure 2. The control samples (treated with YG medium) of all species showed bright green fluorescence as a result of the hydrolysis of FDA by living cells. The treatment by AnCF completely eliminated the fluorescence in the case of *B. cinerea*, *E. necator* and *G. bidwellii* conidia indicating the absence of hydrolytic activity, while *P. viticola* sporangia were not affected.

The effects of AnCF on conidia and mycelia

For the investigation of mode of action of AnCF against fungal cells, *B. cinerea* and *G. bidwellii* were chosen as test organisms, since they can be easily maintained by *in vitro* cultivation in contrast with the biotrophic species. Germination tests were carried out with spores treated with AnCF or YG medium as a negative control. The presence of AnCF drastically decreased the rate of

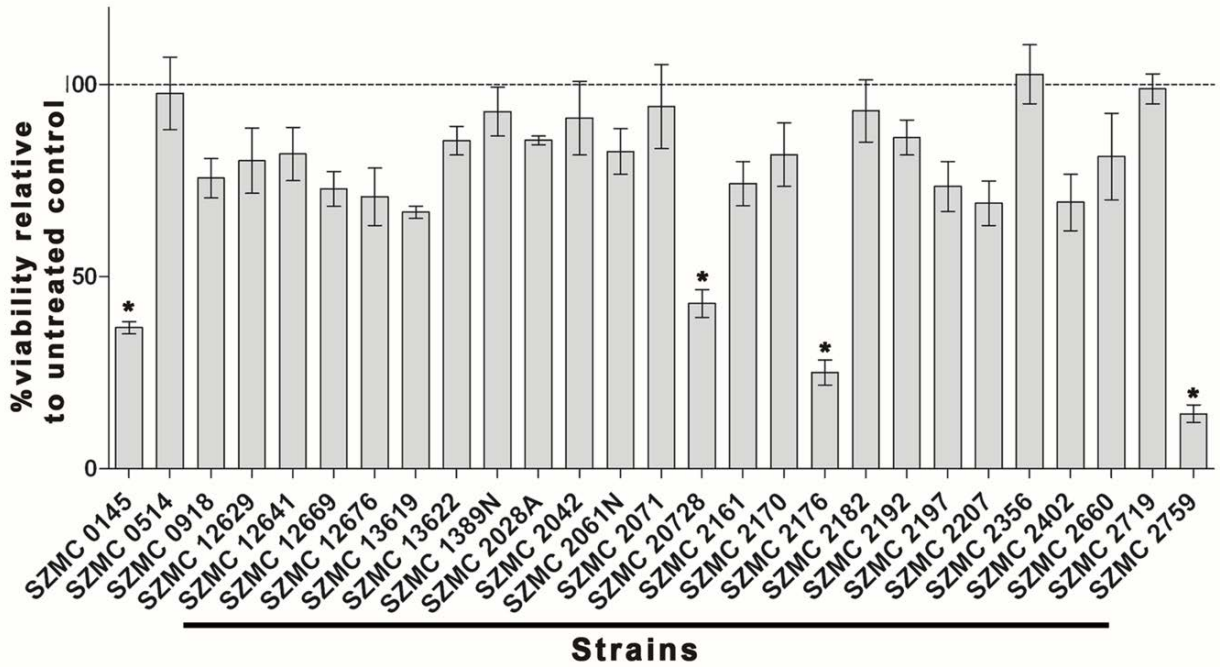

Figure 1. Antifungal activity of fungal culture filtrates on *G. bidwellii* mycelia assessed by thiazolyl tetrazolium bromide assay. The indicated values represent percental viability of treated mycelia relative to control sample, which treated with YG medium. Culture filtrates of fungal strains that caused more than 50% decrease in the viability (SZMC0145, SZMC20728, SZMC2176, SZMC2759) are marked with asterisk. Indicated values represent the average of 3 measurements, error bars show standard deviances

germinating conidia. In case of the control conidia of *G. bidwellii* 82±9.8% of the spores were able to germinate, while only 5±4.6% of the treated conidia developed germ tubes. The same phenomenon can be observed in the case of *B. cinerea* conidia. As the result of the treatment with AnCF 0% of conidia produced germ tubes, while this value was 78±2.5% in the case of YG-treated control.

To reveal the mechanisms behind the antifungal effect of AnCF, several microscopic examinations were carried out with AnCF-treated and untreated *G. bidwellii* and *B. cinerea* mycelia (Figure 3). Plasmolysis was frequently detected in treated mycelial cells (Figure 3A, panel f; Figure 3B, panel f) by crystal violet staining, while the untreated cells were able to maintain their intracellular pressure (Figure 3A, panel a; Figure 3B panel a).

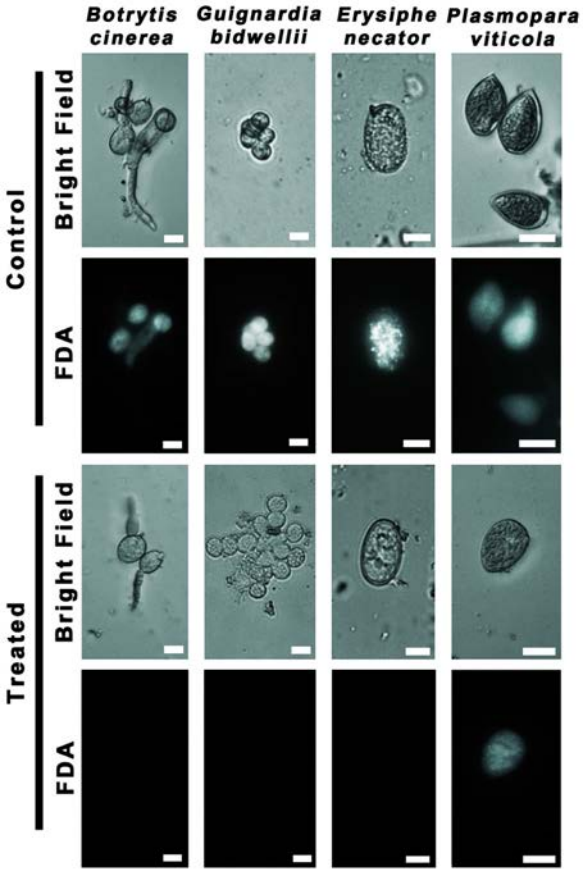

Figure 2. Effect of AnCF on the viability of asexual spores of *B. cinerea*, *G. bidwellii*, *E. necator* and *P. viticola* examined by fluorescein diacetate staining and fluorescent microscopy. The viability of cells treated with YG medium (Control) or with AnCF (Treated) are indicated by the fluorescence of the hydrolysed dye. Scalebars represent 10 µm

SYBR GREEN I staining of untreated (Figure 3A, b and c panels; Figure 3B, b and c panels) and AnCF-treated mycelia (Figure 3A, g and h panels; Figure 3B, g and h panels) revealed that as a result of the treatment with AnCF, the mycelial nuclei were frequently fragmented. Cell membrane permeability of control and AnCF-treated mycelia was investigated by EtBr exclusion and microscopic observations. Mycelia treated with AnCF (Figure 3A, i and j panels; Figure 3B, i and j panels) could uptake EtBr, while the untreated control showed no fluorescence (Figure 3A, d and e panels; Figure 3B, d and e panels) in the case of both *G. bidwellii* and *B. cinerea*.

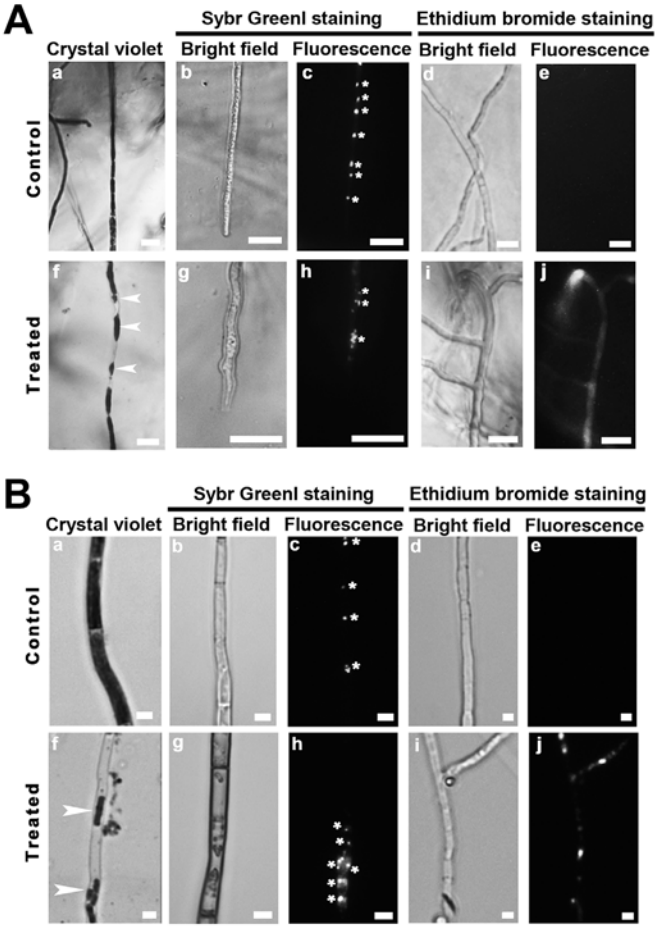

Figure 3. A: Microscopic observation of *G. bidwellii* mycelia treated with YG medium (Control) or with AnCF (Treated). Mycelia were stained for cytoplasm (Crystal violet; a, f), nuclei (SYBR Green I staining; b, c, g, h) and for membrane permeability (Ethidium bromide staining; d, e, I, j). B: Microscopic observation of *B. cinerea* mycelia treated with YG medium (Control) or with AnCF (Treated). Mycelia were stained for cytoplasm (Crystal violet; a, f), nuclei (SYBR Green I staining; b, c, g, h) and for membrane permeability (Ethidium bromide staining; d, e, I, j). Arrowheads mark plasmolysed cells, asterisks mark nuclei. Scalebars represent 10 µm

Antifungal effect of AnCF on infected leaf disks

Viability staining with FDA and subsequent microscopic studies were carried out with leaf discs showing symptoms of black rot (*G. bidwellii*) or powdery mildew (*E. necator*) infections. Results are presented in Figure 4. Both untreated mycelia of *E. necator* (Figure 4b) and *G. bidwellii* (Figure 4a) showed green fluorescence, indicating viable cells. As a result of the AnCF treatment, the fluorescein signal disappeared from *E. necator* mycelia, only a weak autofluorescence could be detected (Figure 4d). However, the treatment did not affect the viability of *G. bidwelii* cells (Figure 4c).

Chemical properties of the active fungicide agent of AnCF

The antifungal activity of AnCF is localized in the solid fraction after TCA precipitation and mostly restored its activity after re-solubilization (Figure 5a). The activity of the antifungal agent was decreased significantly by the application of 2-ME treatment (Figure 5a). The approximate molecular weight of the active agent of AnCF was investigated by non-reducing SDS-PAGE and bioautography (Figure 5b). The molecular weight of the antifungal agent fell into the range between 3 and 6 kDa.

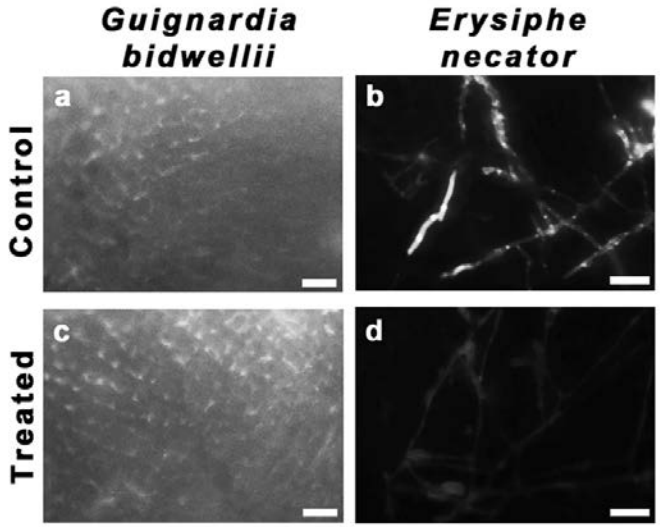

Figure 4. Fluorescent microscopic examination of mycelia treated with YG medium (Control) or AnCF (Treated) in case of *G. bidwellii* (a, c) or *E. necator* (b, d) on leaf discs. Samples were stained with fluorescein diacetate (FDA). The fluorescence of hydrolysed FDA indicates viability. Scalebars represent 100 µm

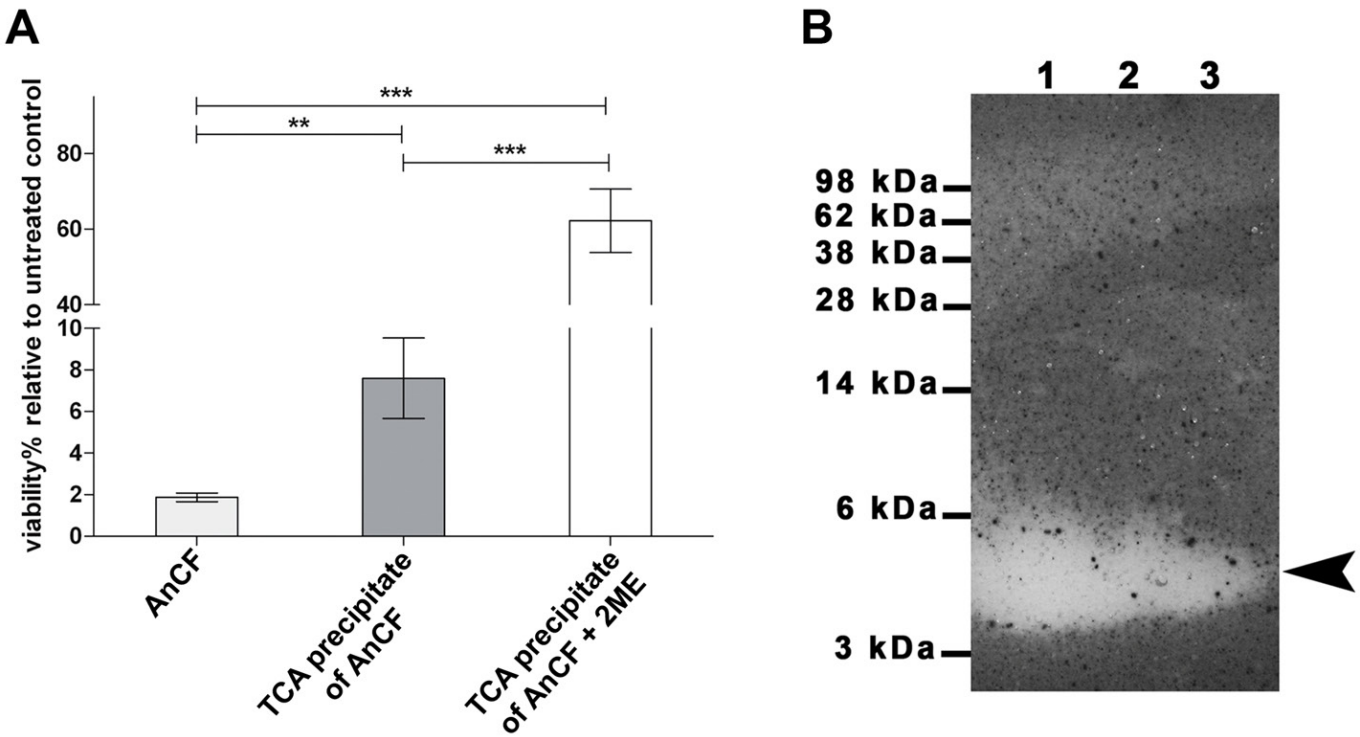

Figure 5. Characterization of the antifungal component of AnCF. A: Effect of trichloro acetic acid (TCA) precipitation and 2-mercaptoethanol (2ME) treatment on the antifungal activity of AnCF against *G. bidwellii* conidia, measured by fluorescein diacetate hydrolysis assay. Indicated values represent the average of 3 measurements, error bars show standard deviance values. Asterisks mark the significance of differences (** p<0,005, *** p<0,0005). B: Results of non-reducing gel electrophoresis of AnCF (lane 1: 40 µl, lane 2: 10 µl, lane 3: 5 µl) and subsequent bioautography with *S. cerevisiae*. The positions of molecular weight marker proteins are labelled on the left. Arrowhead marks the zone of inhibition detected by thiazolyl tetrazolium bromide staining

Optimization of fermentation conditions for AnCF

The effect of certain parameters of cultivation on the antifungal activity of AnCF was tested by FDA assay on *G. bidwellii* conidia. Fermentations were carried out with different initial pH of the fermentation medium (Figure 6a). Between pH 4-6 AnCF caused ~70% loss in the viability of conidia. Above pH 6 increased antifungal activity was observed (up to 93% at pH 8). The effect of aeration was also significant (Figure 6b). AnCF prepared with dynamic conditions had a MIC_{50} (minimal concentration for 50% inhibition) value of 1 v/v%, while this value for AnCF prepared with static condition was 12.5 v/v% (values represent the volumetric rate of AnCF in the sample mixtures). AnCF prepared under the optimized conditions (YG medium, pH 8, 180 rpm stirring) was tested also against *B. cinerea* and *E. necator* by FDA-hydrolysis assay. The MIC_{50} values were 2.4 v/v% for B. cinerea and 2.9 v/v% for *E. necator*. In the presence of osmotic (1 M NaCl) or membrane stressors (0.3 v/v% Tween 80) in the fermentation medium the antifungal activity of AnCF did not altered significantly (data not shown).

Antifungal effect of AnCF on grapevine cuttings

Grapevine cuttings were artificially infected with *E. necator* conidia and were sprayed regularly with AnCF prepared with optimal fermentation conditions or with YG medium (control). After four weeks of incubation all of the control plants showed the symptoms of powdery mildew (white spots of mycelia on leaves) with varying severity (Figure 7a) The AnCF-treated plants did not show the symptoms of powdery mildew or any negative effect, which would be caused by AnCF (Figure 7b).

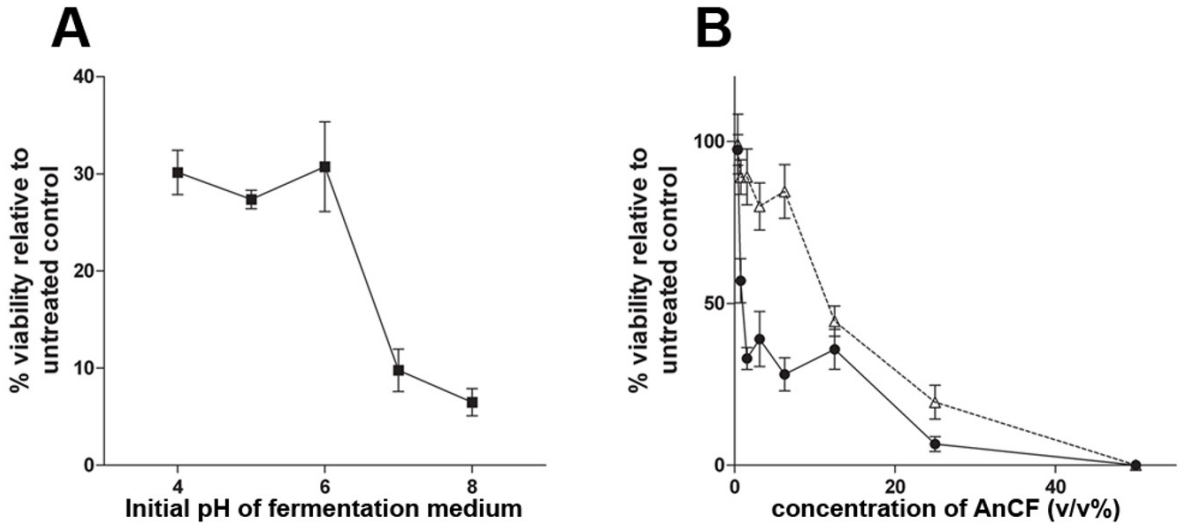

Figure 6. The effect of initial pH of the fermentation medium (A) and aeration (B) on the antifungal activity of AnCF against *G. bidwellii* conidia by quantitative fluorescein diacetate hydrolysis assay. On panel B, open triangles represent fermentation with static condition, closed circles represent dynamic condition (stirring with 180 rpm). Indicated values represent the average of 3 measurements, error bars show standard deviances

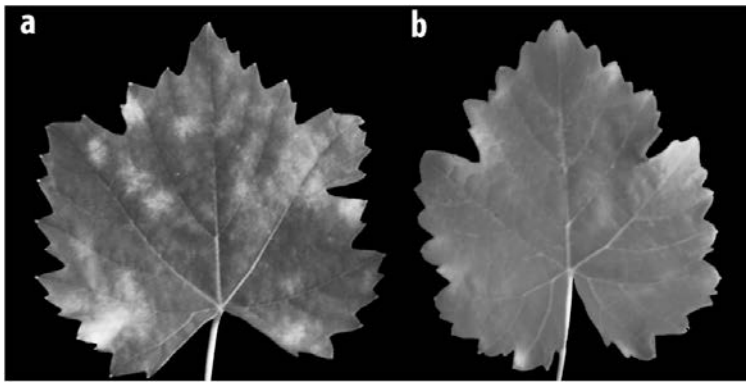

Figure 7. Leaves of Kékfrankos grapevine cuttings artificially infected with *E. necator* conidia and incubated in greenhouse for four weeks. Plants treated with YG medium (a) or with AnCF (b) weekly

DISCUSSION

In the present study a range of culture filtrates of potential defensin-producing fungal isolates were tested for antifungal activity. One *A. niger* strain (SZMC2759) out of the four isolates showing the highest antifungal potential (one *A. clavatus* and three *A. niger*) was examined in details. The spectrum of susceptible pathogenic species of grapevine, mode of action, optimal fermentation parameters, chemical characteristics of the active agent, *in vitro* and in planta antifungal effects were investigated.

The active component of AnCF proved to be a low molecular weight, TCA-precipitable compound with sensitivity to 2-ME (Figure 5). The TCA-precipitable nature of the active compound suggest that it is a protein, and the sensitivity to 2-ME indicates that it contains disulphide bonds. These above results suggest that the active antifungal agent of AnCF is the ANAFP defensin (Gun Lee et al., 1999) of *A. niger*. The measured molecular weight of the active agent was lower than the molecular weight of ANAFP (10 kDa). This latter result may be explained by the combination of the disulphide bonds -which stabilize the structure of ANAFP- and the non-reducing condition of the electrophoresis, which latter was necessary for the recovery of antifungal activity after electrophoresis. The protein without the reduction of the disulphide bonds retained its compact structure, which resulted in a higher electrophoretic mobility.

The susceptibility tests have proven that AnCF is an effective fungicide not only against *G. bidwelli* but *B. cinerea* and *E. necator* as well. AnCF decreased the viability of the treated cells by 50% in high dilutions (1-3 v/v%), but it was not toxic on sporangia of *P. viticola* a pseudofungal species. The ineffectiveness of AnCF against *P. viticola* can be explained by the large taxonomical distance between the oomycetous *P. viticola* and the tested ascomycetous fungi.

The present study on the mode of action of AnCF have revealed mechanisms already known concerning different defensins but have not been investigated yet in the case of ANAFP, the proposed active agent of AnCF. Plasmolysis frequently occurred in the treated *G. bidwellii* and *B. cinerea* cells (Figure 3A, panel f; Figure 3B, panel f). This phenomenon is not lethal on its own, but it decreases the fitness of fungal cells and prevents the proliferation at the mycelial tips (Bitsikas et al., 2011). This result would be the first observation of defensin-induced plasmolysis in fungi. However, this theory needs further verification by the use of purified ANAFP. A similar observation was only reported in the case of *Escherichia coli* cells treated by human β-defensin 2 (Estrela et al., 2013). Treatment with AnCF caused the permeabilization of the fungal cell membrane as well (Figure 3A, i and j panels; Figure 3B, i and j panels). Cells with impaired plasma membrane cannot maintain the optimal intracellular environment and inevitably die. The same phenomenon was detected in the case of *A. giganteus* AFP and *P. chyrsogenum* PAF defensins (Theis et al., 2003; Leiter et al., 2005). Fragmented nuclei were observed in a significant proportion of mycelial cells treated with AnCF (Figure 3A, g and h panels; Figure 3B, g and h panels). These particles are looked similar to apoptotic bodies (Cao et al., 2012). These results suggest the presence of an apoptosis-like mechanism, which was previously observed in *Aspergillus nidulans* when treated with *P. chrysogenum* PAF defensin (Leiter et al., 2005). The several mechanisms of action suggest that AnCF could be applied without the risk of the development of resistance in the targeted fungi.

The fungicide tests carried out on *E. necator* and *G. bidwellii*-infected leaf discs showed a limitation of the AnCF as a spray (Figure 4). The agent is not able to penetrate the leaf cuticle, therefore cannot be applied as a curative fungicide against subcuticular mycelia. However, its high efficiency against unprotected fungal cells (epicuticular mycelia, fruiting bodies and spores) can be useful in the prevention of subcuticular infections.

Preliminary, laboratory-scale experiments were carried out for the optimization of fermentation conditions for high antifungal efficacy (Figure 6). A simple complete medium (YG) with a slightly alkaline pH (pH 8) and aerated conditions were sufficient for the preparation of a culture filtrate, which was effective in high dilutions. However, further industrial-scale experiments are needed for the

optimization of the cost-effective production of this fungicide.

In vitro experiments cannot show the actual applicability of a fungicide. The unpredictable interactions between the host, the pathogen, the environment and the fungicide can lead to loss in the antifungal effect. To investigate the possible effects of the grapevine on the antifungal effect of AnCF, *in vivo* experiments were carried out on grapevine cuttings artificially infected with *E. necator*. The application of AnCF prevented the formation of foliar symptoms of powdery mildew, while all the control plants (sprayed with YG medium) showed these symptoms (Figure 7). These results suggest that the active fungicide component of AnCF is not inhibited by the action of the plant (e.g. by proteolytic cleavage). Moreover, the treated plants did not show any negative effect of AnCF on grapevines. These results support that AnCF would be used in field conditions with high reliability.

CONCLUSIONS

In the present study it is demonstrated that the culture filtrate of *A. niger* could be used for the control of some important fungal pathogens of grapevine. The agent exhibited high antifungal activity against important pathogens of grapes such as *B. cinerea*, *E. necator* and *G. bidwellii*. However, the results also pointed out some limitations of AnCF as a spray against fungal and pseudofungal infections of grapevine. The active agent of AnCF cannot penetrate in the leaf cuticle and did not affect *P. viticola* cells. Optimization of some parameters of fermentation was done, however further examinations should be carried out. Nevertheless, the widespread use of *A. niger* in the fermentation industry, suggests the cost-effective production of the antifungal agent as a side product of widely used fermentation processes.

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