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STUDYING THE ACTIVITY OF *Chenopodium album* SEED EXTRACTS AND *Fusarium sambucinum* CULTURE LIQUID AGAINST SEVERAL PLANT PATHOGENIC FUNGI

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Abstract

Plant extracts and microbial culture liquids contain a great number of bioactive substances, including potential biofungicides, which are effective against plant diseases. Screening of extracts and cultural filtrates for ability to suppress several pathogenic fungi that damage various agricultural plants can result in discovery of new compounds with a broad range of fungicidal activity. This work is the first report on in vitro testing the activity of plant extracts, which obtained by serial extractions of *Chenopodium album* seeds with hexane followed by ethyl acetate and then with ethanol, and also *Fusarium sambucinum* culture liquid filtrate (CLF), against six widespread fungi (*Alternaria alternata*, *A. dauci*, *A. radicina*, *Bipolaris sorokiniana*, *Septoria tritici*, *Stagonospora nodorum*) that are strongly pathogenic for several economically important crops. These fungi were found to demonstrate different sensitivity to CLF and seed extracts. Thus, CLFs with the activity level that was shown earlier for *Stagonospora nodorum* and *A. radicina* (effective dilutions up to 1:200 и 1:5, respectively) fully inhibited spore germination of all pathogens except *B. sorokiniana*, saving their antifungal activity to dilutions 1:5 (for *A. dauci*) or 1:50 (for *Septoria tritici*). Seed extracts possessed no toxicity against *Stagonospora nodorum*, *Septoria tritici*, *A. dauci* and *A. radicina*, but some of them reduced *A. alternata* and *B. sorokiniana* spore germination. For instance, after seed extraction with hexane followed by ethyl acetate, the number of germinated *B. sorokiniana* spores decreased by 66 % as compared to control. Hyphae of spores germinated in diluted CLF were morphologically defective and much shorter than control ones. Besides, a significant growth retardation of *Stagonospora nodorum*, *Septoria tritici* and *B. sorokiniana* mycelia was observed on agar media supplemented with CLF (100 µg/ml). Collectively, these results suggest that further research of CFL and the seed extracts can result in identification of antifungal metabolites, which could be promising as biofungicides against leaf and glume blotches of wheat, spot blotch or common root rot of other cereals, and *Alternaria* diseases of carrot.

Keywords: *Chenopodium album*, *Fusarium sambucinum*, fungitoxicity, biofungicides, glume and leaf blotches, spot blotch, common root rot, *Alternaria* diseases

Plants and microorganisms produce many biologically active compounds, which functions are the subject of intense research. In particular, one of the modern trends in agricultural science is seeking for active ingredients to create biofungicides and other biopesticides [1-3] as an alternative or an adjunct to available chemical pesticides [4-8]. These promising compounds of microbial or plant origin include antimicrobial peptides and other secondary metabolites [9-11]. Their most accessible sources are extracts of wild (often weed) plants, as well as the culture liquid (CL) of fungi and bacteria grown in vitro [12-17].

Moreover, plant extracts and culture filtrates with pesticidal activity may be used to treat plants without additional (sometimes cost and complicated) purification [18-21]. For example, it was shown that the methanol extracts of lamb's quarters (*Chenopodium album*) inhibit accumulation of *Macrophomina phaseolina* fungus biomass in submerged culture [22], and a non-phytotoxic CL

of the FS-94 isolate of *Fusarium sambucinum* Fuckel effectively inhibits in vitro germination of spores in causal agents of Stagonospora glume blotch and black rot of carrots [16, 17).

It is obvious that a particular interest for crop protection is in searching for extracts and filtrates, able to simultaneously suppress the development of several fungi pathogenic to various crops.

In view of this, we for the first time investigated the activity of the extracts of *Chenopodium album* seeds and CL filtrates of a non-pathogenic *Fusarium sambucinum* against six common plant pathogenic fungi (*Alternaria alternata*, *A. dauci*, *A. radicina*, *Bipolaris sorokiniana*, *Septoria tritici*, *Stagonospora nodorum*), which cause serious damage to many economically important crops.

Techniques. To prepare plant extracts, 30 g of lamb's quarters (*C. album*) seeds were powdered and extracted sequentially with hexane, ethyl acetate and ethanol (a ratio for each solvent 1:8 w/v) under vigorous stirring for 1 h at room temperature. After each extraction step, the homogenate was centrifuged at 10,000 g and at 4 °C for 10 min, the supernatant was filtered through a paper filter and evaporated to dryness in a rotary evaporator. The precipitate was stored at -80 °C and dissolved immediately prior to experiments in a minimal volume of 5 % dimethyl sulfoxide (DMSO).

The culture liquid filtrate (CLF), obtained by submerged cultivation of the FS-94 isolate of *F. sambucinum* (16), was purified from culture medium components and low molecular weight exometabolites, not assimilated by the fungus, by means of ultrafiltration fractionation [23], while washing the CLF concentrate with sterile distilled water (sdH₂O). Preparations of the concentrates transferred into aqueous medium were adjusted with sdH₂O up to a volume of the initial CLF, sterilized by filtration through a 0.22 µm membrane (GPWP type, MilliporeSigma, USA), frozen and stored until use at -20 °C.

Alternaria alternata (Fr.) Keissl; *Bipolaris sorokiniana* (Sacc.), Shoemaker; *Septoria tritici* Desm. and *Stagonospora nodorum* Berk., Castellani & E.G. Germann isolates were obtained from the National Collection of Phytopathogenic Microorganisms in the All-Russian Research Institute of Phytopathology, and *A. dauci* (J.G. Kuhn), J.W. Groves & Skolko and *A. radicina* Meier, Drechsler & Eddy isolates were from the Institute for Breeding Research on Horticultural Crops, Germany. To prepare suspensions, on the day of the experiment fungal spores, using sdH₂O, were washed off the surface of the colonies grown for 14 days on PDA at 25 °C (*A. alternata*, *B. sorokiniana*, *Stagonospora nodorum*, *Septoria tritici*) or on vegetable broth agar medium at 22-23 °C (*A. dauci*, *A. radicina*). Spores were separated from mycelial fragments by filtering through cotton and nylon filters, then pelleted by centrifugation at 3,000 g for 20 min and resuspended in a minimal volume of sdH₂O.

To evaluate the plant extracts and CLF for fungitoxicity, a method of spore germination in a thin layer of agar was used. To do this, equal volumes of the initial suspension and the test samples were mixed so that the density of a final suspension was 10⁴/ml for *A. alternata*, *A. dauci*, *A. radicina* and *B. sorokiniana* or 3×10³/ml for *Stagonospora nodorum* and *Septoria tritici*. The suspensions were incubated at a constant slow stir for 25-30 minutes at room temperature and then applied onto slides coated with 1 % aqueous agar (200 µl per slide, 3 slides per each test option), which were placed in a moist chamber. The spores were germinated in the dark at 22 °C for 16-18 (*A. dauci*, *A. radicina*, *Stagonospora nodorum*, *Septoria tritici*) or 5-6 hours (*A. alternata* и *B. sorokiniana*). As a control we used spore suspension in sdH₂O in the experiments with CLF, and in sterile 2.5 % DMSO for the analysis of the *C. album* extracts. The effects in-

duced by CLF and extracts on germination of plant pathogens were evaluated by microscope, scanning at least 600 spores in each treated option and the control. Germination frequency was expressed as a percentage relative to either a number of spores germinated in the control or a total number of spores, scanned in the control and in the experimental options.

To evaluate the effect of CLF on the radial growth of colonies of *Stagonospora nodorum*, *Septoria tritici* и *B. sorokiniana*, they were grown for 14-16 days in the dark at 25 °C on the PDA containing the filtrate (100 µl CLF/ml culture medium). The mycelium fragments were used as the inoculum to be spiked to the center of Petri dishes (9 cm in diameter). Control cultures were grown under the same conditions on media without the addition of CLF. As cultures grew, the diameter of the colonies (2 measurements per each colony in 2 different directions, 3 dishes per test option) was measured and the average diameter of the colonies for each of the pathogens was calculated.

Tests on the spore germination and the study of the growth trends in the plant pathogens were repeated at least 3 times. Statistical processing of the results was performed using Statistica 6.0 software (SoftStat Inc., USA). The significance of differences vs. control ($p \leq 0.05$) was determined using *t*-test for independent variables.

Results. As we have previously found that the CLF was capable of inhibiting the germination of *Stagonospora nodorum* and *A. radicina* spores [16, 17], in this study we used these plant pathogens as the reference test objects for monitoring the activity of the resulting filtrates when evaluating their fungitoxicity against *Septoria tritici*, *A. dauci*, *A. alternata* and *B. sorokiniana*. These species are causative agents of dangerous diseases of important agricultural crops, such as leaf and glume blotches of wheat (*Septoria tritici*), barley leaf spot and common root rots of the grasses (*B. sorokiniana*), carrot leaf blight (*A. dauci*), dark mildew of wheat ears, as well as blackspots of a variety of other plants (*A. alternata*) [24-27].

1. Comparative fungicidal activity of the culture liquid filtrate (CLF) of *Fusarium sambucinum* against *Septoria*, *Helminthosporium* and *Alternaria* pathogens

Pathogen	Germination of spores in the CLF, % of control	The CLF titer
<i>Stagonospora nodorum</i>	0	1:200 ($p = 0.02$)
<i>Septoria tritici</i>	0	1:50 ($p = 0.01$)
<i>Bipolaris sorokiniana</i>	98.3±1.3	No dilution
<i>Alternaria radicina</i>	0	1:5 ($p = 0.03$)
<i>A. alternata</i>	5.0±0.80	1:5 ($p = 0.02$)
<i>A. dauci</i>	2.0±0.04	1:5 ($p = 0.04$)

Note. The table presents the average values for 600 spores per each test option and a standard error (mean±SE) and the maximum CLF dilutions, with which there was still significant ($p = 0.05$) inhibition of spore germination vs. control.

The tests have shown that the CLF with a typical fungicidal activity against the reference test objects (the absence of germinated spores in undiluted filtrates and the expected titers of 1:200 and 1:5, respectively, for *Stagonospora nodorum* and *A. radicina*) had a similar activity against *Septoria tritici*, *A. alternata* and *A. dauci*, and the inhibitory action of the CLF against *S. tritici* was more effective, although this species was 4 times less sensitive than the other causative agent of septoria wheat, i.e. *Stagonospora nodorum* (Table 1). As in the case with *A. radicina*, a statistically significant inhibition of germination of *A. alternata* and *A. dauci* spores was observed up to 5-fold dilution of the CLF, and in the initial filtrates the germination was inhibited almost completely. The fungus *B. sorokiniana* appeared to be resistant to the effect of CLF.

Note that all the investigated plant pathogens, including *B. sorokiniana*,

hyphae of spores, which retained the ability to germinate after exposure to CLF, had morphological defects, particularly marked thickenings, and were significantly shorter than in control (Fig. 1).

Fig. 1. *Bipolaris sorokiniana* spores, germinated in sterile distilled water (left) or in the culture liquid filtrate of *Fusarium sambucinum* (right).

Fig. 2. Trends in growth of *Stagonospora nodorum* (A), *Septoria tritici* (B) and *Bipolaris sorokiniana* (C) on potato dextrose agar (PDA) containing the culture liquid filtrate (CLF) *Fusarium sambucinum*: 1 — control (PDA without CLF), 2 — PDA with CLF (100 µg/ml). The percentage of inhibition of radial growth of colonies is specified. Different starting dates of measurements are related to different rates of fungi growth. Y-errors indicate

mean deviations for two experiments (6 replicates per test option per each experiment).

An analysis of the growth trends for the *Stagonospora nodorum*, *Septoria tritici* and *B. sorokiniana* mycelium in the presence of CLF revealed that, when cultured in media containing the filtrate, these plant pathogens markedly decreased their colony size (Fig. 2). The observed fungistatic effect on the growth of *Stagonospora nodorum* and *Septoria tritici* was stable and changed only slightly by the end of cultivation. In contrast, the inhibitory effect of the CLF on the growth of *B. sorokiniana* mycelium gradually weakened, however, the differences vs. control remained until the completion of observations (see Fig. 2).

Testing of preparations obtained by sequential extraction of *C. album* seeds using hexane, ethyl acetate and ethanol demonstrated that none of them possessed fungitoxicity against *Stagonospora nodorum*, *Septoria tritici*, *A. dauci* and *A. radicina*. Meanwhile, some of these plant extracts were able to inhibit to different extents the germination of the *A. alternata* and *B. sorokiniana* spores (Fig. 3). Thus, incubation of the *A. alternata* spores in the extracts obtained using hexane resulted in significant reduction in the number of germinated spores (by 16 %), and further decreased it in the ethanol-based extracts by 14 % more.

The number of the *B. sorokiniana* spores, which germinated after incubation in extracts using hexane and ethyl acetate, was reduced by 34 and 32 %, respectively. Therefore, combined inhibition of germination using *C. album* ex-

tracts for these plant pathogens reached 30 % (*A. alternata*) and 66 % (*B. sorokiniana*). Interestingly, that if *B. sorokiniana* concerned, the fungitoxicity effect, observed in two of the three experiments on the concurrent use of the CLF and preparations after extraction with hexane or ethyl acetate (Table 2), according to Limpel's formula ($E_0 < E_{real}$), had the synergistic action [28]. Furthermore, the estimated excess of the additive inhibitory effect in case of a combined use of the filtrate and both of the sequentially produced extracts amounted to 27-28 %.

Fig. 3. Germination of the *Alternaria alternata* (A) and *Bipolaris sorokiniana* (B) spores when incubated with the preparations, obtained from the seeds of lamb's quarters (*Chenopodium album*) by sequential extraction with hexane (a), ethyl acetate (b) and ethanol (c). The extracts were dissolved in 2.5 % dimethyl sulfoxide (DMSO); C — control (2.5 % DMSO). The spores were placed in the studied extracts. To calculate the percentage of germinated spores (mean of 3 experiments), at least 600 spores per test option in each experiment were scanned

after 5-6 hours. Standard errors of mean are given.

2. Inhibition of spore germination (% of total) in *Bipolaris sorokiniana* in a combined use of the *Fusarium sambucinum* culture liquid and *Chenopodium album* seed extracts

CLF	EH	EEA	CLF + EH		CLF + EEA	
			E_0	E_{real}	E_0	E_{real}
0.8	36.3	31.9	37.1	49.6	32.7	47.2
1.3	34.7	29.6	36.0	48.5	30.9	46.7
0.6	31.0	34.2	31.4 ^a	31.9 ^a	34.8 ^b	35.2 ^b

Note. EH, EEA — extraction with hexane or ethyl acetate, respectively; titer of culture liquid filtrate (CLF) 1:2. E_0 — expected additive effect, %; $E_0 = (X + Y) - XY/100$, where X — CLF, Y — EH or EEA. E_{real} — Effect (%) obtained in the experiment. The differences between E_0 vs. E_{real} are significant at $p = 0.05$, except for the values designated with the same Latin letters. If $E_0 < E_{real}$ (28), the inhibitory effect is synergistic.

Thus, our results on evaluating the influence of the *Fusarium sambucinum* culture liquid and *Chenopodium album* seed extracts on in vitro development of six pathogenic fungi suggest a promising outlook of further research devoted to the composition of these products aimed at identifying the metabolites responsible for the antifungal activity, as well as to assume the possibility of creating biopesticides based on them against leaf and glume blotches of wheat, *Alternaria* diseases of carrots and spot blotch of the grasses.

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