БИОЛОГИЧЕСКИЕ НАУКИ

EFFECT OF MARINE FUNGAL SECONDARY METABOLITES ON PLANT ROOT GROWTH

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ABSTRACT. Growth-regulating activity of 103 crude ethylacetate extracts of marine fungal isolates and nine individual compounds isolated from fungal cultures was determined by the treatment of buckwheat seeds as biological test-object. For evaluation of growth-regulating activity of the extracts tested, the technique of germinating seeds in Petri dishes on filter paper and in rolls of filter paper was used. Plant growth-regulating activity of crude fungal extracts was strongly strain-dependent. Sixteen crude fungal extracts of *Aspergillus recifei*, *Penicillium brevicompactum, P. chrysogenum, P. islandicum, P. restrictum, Beauveria bassiana, B. brongniartii* isolates exhibited the most inhibition effect on buckwheat germs root growth (50-70 %). Sterigmatocystin isolated from *A. versicolor* inhibited buckwheat root growth by 32 %. Six crude extracts of fungal strains belonging to the species *Cosmospora butyri, P. bilaii, A. fuci, A. recifei* had the slight growth stimulation effect (20 %). The data about effect of austalids, diorcinols and sterigmatocystin on plant root growth have been obtained for the first time. It was shown that marine fungi could be used as potential sources of compounds with a plant growth-regulating activity.

Keywords: buckwheat germs, ethylacetate extracts, growth-regulating activity, marine fungi, sterigmatocystin

Introduction

Fungi are an integral part of practically all biocenoses of our planet including marine ones [1, 4, 5, 7]. Fungi of marine habitats as well as fungal associates are sources of various biologically active compounds of different chemical nature, namely: alkaloids, glycosides, terpenoids, carotenoids, prostaglandins, which possess high antibacterial, antifunginal, antitumor, hemolytic and other types of activity [3, 6]. The part of marine fungi metabolites studied contain biologically active compounds of new chemical structure. Discovering compounds, which have growth regulating activity, is important not only from scientific viewpoint but has wide practical significance. Inhibiting and stimulating root-forming activity of compounds is, undoubtedly, important for agriculture and forestry as well as for cultivation of medical plants. Growth regulating activity of marine fungal metabolites is practically not studied.

We searched producers of substances with plant growth regulating activity among fungi isolated from marine habitats for the first time. Use of fungi strains as producers of biologically active compounds, which influence seeds germination activity, has one more important economic aspect that is a cheap method of producing biologically active substances as well as an alternative ecologically safe microbiological method of growth regulation of plants in nature conservancy zones where using chemical agents is absolutely prohibited.

Materials and methods

Specimen collection and identification. Objects for discovering producers of substances, which have growth regulating activity, were 103 strains of fungi extracted from samples of sea ground of various areas of Okhotsk Sea. Samples collection was made during the scientific and research voyage No. 29 aboard the research vessel «Akademik Oparin» in 2004. Material was collected by the method of dredging with the use of Van Veen grab with grasp width 0.5 m. The samples were stored in sterile polyethylene bags, in refrigerating plants under the temperature – 12 °C. Fungi isolation was carried out according to the standard method, to agarized ground decoction in sea water prepared according to the method of soil decoctions and agarized

beer wort in sea water with antibiotics added (500 thousand units of penicillin and 0.5 g streptomycin per liter), by the method of culturing and direct planting of ground. For fungi identification, the following media were used: agarized beer wort in sea water, Czapek's medium, Bilai's medium. For identification of fungi isolated, common keys and guides were used. For confirmation of the species level, the PCR method and the following pairs of primers appropriate to the genes locus ITS (1), beta-tubulin (2), calmodulin (3) (additionally for *Penicillium* spp.), actin (4) (additionally for *Cladosporium* spp.) were used:

(1) ITS1 - 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 - 5'-TCCTCCGCTTATTGATATGC-3';
(2) Bt2a - 5'-GGTAACCAAATCGGTGCTGCTTTC-3'and Bt2b - 5'-ACCCTCAGTGTAGTGACCCTTGGC-3';
(3) Cmd5 - 5'-CCGAGTACAAGGAGGCCTTC-3' and Cmd6 - 5'-CCGATAGAGGTCATAACGTGG-3';
(4) act-512F - 5'-ATGTGCAAGGCCGGTTTCGC-3'and ACT-783R - 5'-TACGAGTCCTTCTGGCCCAT-3'

Strains cultivation and extraction. For cultivation of fungi strains, slant agarized wort with the following composition was used: sea water – 800 ml; liquid beer wort – 200 ml; agar – 20 g, pH 7.8 - 8.2. Cultivation were performed during 2 weeks under the room temperature. Fungi cultures were kept with the use of the same medium. For derivation of fungal secondary metabolites, the fungal strains cultures were extracted with ethylacetate. For this purpose, a fungus culture was inoculated into the tube with slant agarized medium and cultivated. On the 14th day of fungi mycelial cultures growth about 10-15 ml of ethylacetate were added into each tube, and they were kept under the room temperature within 12 h. Ethylacetate extract was filtered through paper filter and boiled out on rotary evaporator. Extraction was carried out twice. Use of ehtylacetate allows limiting the search of biologically active compounds by low-molecular area. Sterigmatocystin was isolated from marine-derived fungus *Aspergillus versicolor* KMM 4644 as describe by [8]. Austalides H, I, J, P were isolated from the alga-derived fungus *Penicillium thomii* KMM 4645 as described by [9]. Diorcinol B and D were isolated from sediment-derived fungus *Aspergillus sulphureus* KMM 4640 as describe by [10].

Growth-regulating activity assay. Growth-regulating activity of crude extracts from marine fungi was determined according to the method described by [2]. Fungi extracts boiled out dry were solved in 500 µl of ethylacetate. Then, 50 µl of the solution obtained was diluted up to 5 ml by distilled water to the final solution concentration of active substances was about 300 µg ml^{-1}. The solutions obtained were used for determination of growth regulating activity. As biological test-objects, the seeds of buckwheat *Fagopyrum esculentum* Moech of "Izumrud" variety harvested in 2014 are used. For evaluation of growth regulating activity of the extracts tested, the technique of germinating seeds in Petri dishes on filter paper was used. Dry seeds were put onto filter paper circles (diameter is 9 cm), which had been previously wetted with aqueous solution of the preparation tested, and were incubated within 48 hours in thermostat under 27 ºC. The germs obtained from the seeds steeped in distilled water were the control. For evaluation of biological activity of individual compounds, the technique of germinating seeds in rolls of filter paper was used. Twenty five dry seeds were put onto a filter paper strip (12 cm x 42 cm) wetted with solution of the compound tested under various concentrations (0 - 10 µg ml^{-1}). Strips were folded up and put into glasses with 100 mL solution of the compound tested and were incubated within 3 days under 23-25 °C. The seeds grown in water were used as the control. After incubation, the length of the germs main root was measured. Impact of solutions of the preparations tested on the germs growth was expressed as the ration of the treated plant main root length to the control plant root length and was presented in percent.

Statistics. Tests were carried out for each crude extract or individual compound concentration in triplicate. The plantlet main root length was measured after incubation. The control roots length was taken as 100 %. The results were expressed in percent of the control (M ± SE). The data were analyzed with the use of Origin 7.0 program.

Results and Discussion

When studying fungal metabolite complexes of Okhotsk Sea areas, stimulating activity toward buckwheat germs was shown mainly for fungi species of *Acremonium*; while inhibiting activity - mainly for fungi of *Penicillium*. However, growth-regulating activities of crude fungal extracts were rather strain-dependent (Table 1).

Table 1. Growth-regulating activity of fungal individual compounds

Test object	*Source of extraction*	*Concentration, μg mL^{-1}*			
		0.0	0.1	1	10
		Germs main root length, % to the control			
Sterigmatocystin	*Aspergillus versicolor* (Vuill.) Tirab.	100±3	104±4	94±6	68±4
13-diacetoxy austalid I	*Penicillium thomii* Maire	100±2	110±2	103±2	101±1
Acid of austalid H	*P. thomii*	100±1	105±3	107±2	104±1
Austalid H	*P. thomii*	100±3	101±2	100±2	103±2
Austalid J	*P. thomii*	100±2	100±1	101±2	105±4
Butyl ether of austalid H	*P. thomii*	100±3	100±5	116±3	103±6
Butyl ether of austalid P	*P. thomii*	100±1	98±3	97±3	97±2
Diorcinol B	*A. sulphureus*	100±1	108±4	112±1	110±3
Diorcinol D	*Aspergillus sulphureus* (Fresen.) Thom&Church	100±2	95±2	101±2	106±3

It is known that among biologically active metabolic products of organisms, the substances acting as inhibitors are antibiotics, marasmines, phytoncids, colines, and as stimulants are excitators, gibberellins, catacolines, intercolines that are common and mainly hormone-like substances. Studying metabolites of *Penicillium* fungi, indeed, shows presence of compounds with high antibiotic activity. Data about root stimulating activity of metabolites of *Acremonium* fungi are not presented in literature. It is known that *Acremonium* fungi are usually slow growing fungi, marine isolates of which produce compounds of polyketide nature. However, terrestrial analogues are sources of products with antibiotic spectrum of activity, for example, cephalosporin. In plant industry, antibiotics including those of fungi are used as herbicides, insecticides, plant growth stimulants. High occurrence frequency of strains of fungi-producers with inhibiting activity of substances is fully explainable by the fact that many extracellular low-molecular metabolites of fungi and other microorganisms have inhibiting activity crushing their competitors for a source of feed and, thus, play a significant role in their adaptation. Stimulant property of metabolized substances is mostly specific for associate fungi because between macro- and microorganisms specific symbiotic food chains are forming, which play a great role in life support of a microorganism – fungus. Fungi are often found as associates of sea sponges, echinoderms as well as of seaweeds and sea grass. Exactly fungi from these ecological groups produced more than 50 % of new structure compounds obtained from sea sources. When analyzing the results of testing growth regulating activity of ethylacetate extracts of fungal strains from bottom sediments (Table 1), it can be noted that capacity for producing compounds with growth regulating activity is not a species but a strain character because only specific strains of fungi species mentioned above synthesized growth regulating compounds. Thus, for example, extracts only of two strains of 13 tested *A. fuci* fungi strains (15.39 % of total strains) demonstrated plant growth regulating (stimulating) activity (Table 1). Approximate distribution of activity is also specific for extracts of *A. recifei* fungi strains, 2 of 6 strains (33.33 % of total strains), as well as for extracts of *W. inflatus* strains, two of seven strains (28.57 % of total strains) demonstrated growth regulating (stimulating) activity. This fact is in line with the literature data, in which by the example of antibiotic activity it was also shown that not all microorganisms have capacity to produce antibiotics but only some strains of particular species. In addition, inhibiting activity distribution was unexpected among *B. bassiana* strains of the known entomopathogenic fungus based on which culture the biopreparation has been developed for biological control of insects – Boverin, effective agent of which is the fungus itself and its toxins. From our experimental data, only one of five tested strains of this species inhibited buckwheat root growth by 71 %, inhibition value of other four strains did not exceed 48 %. From now we continued studying growth regulating activity of individual compounds obtained elderly from *P. thomii* [9], *A. versicolour* [8], and *Aspergillus sulphureus* [10] (Table 1). In the process of discovering individual compounds being responsible for growth regulating activity we identified, for the first time, diorcinols B and D for marine isolate of fungus *A. sulphureus* strain KMM 4648 isolated from sea ground (Okhotsk Sea, Deryugin deep, near Northern Sakhalin, 385-m depth, and region of gas and methane outflow, 2009). As it can be seen from Table 2, growth-regulating activity of individual compounds studied was little different from the control samples. Only 4 substances demonstrated more or less significant stimulating effect on buckwheat germs root growth: 13-deacetoxi austalid I in concentration of 0.1 μg ml^{-1} for 10 %; acid of austalid H in concentration of 1 μg ml^{-1} for 7 %; butyl ether of austalid H in concentration of 1 μg ml^{-1} for 16 %; Diorcinol B in concentration of 0.1 μg ml^{-1} for 8 % and in concentration of 1 μg ml^{-1} for 12%. From the compounds tested only one compound can be mentioned as practically significant – sterigmatocystin, which was extracted from *A. versicolor* and demonstrated inhibiting activity towards buckwheat germs root growth. In concentration of 1 and 10 μg ml^{-1} inhibiting effect was 6 and 32 %, respectively (Table 1).

Thus, it has been determined that sterigmatocystin is a significant inhibition agent for buckwheat seeds germs root growth. In addition, it was authentically shown that the use of fungal marine isolates as a potential biological resource could be involved into human

economic activity for regulation of root formation, with domination of inhibiting substances among their secondary metabolism products.

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