Antifungal Potential of Organic Acids Produced by *Mortierella Alpina*

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Abstract

The effect of metabolites is synthesized by *M. alpina* for growth and the synthesis of mycotoxins was studied using phytopathogenic fungi *Purpureocillium lilacinum, Fusarium tricinctum* and *Fusarium oxysporum* on infected rhizosphere and seeds of alfalfa cultivars. It was found that culture filtrate medium is suppressed into colony formation of phytopathogenic fungi from 56 to 96%. Arachidonic acid inhibits the growth of *F. tricinctum* and *F. oxysporum* by 69 to 90%, respectively, and enhanced by 62% in *P. lilacinum.* Moreover, arachidonic acid was found to be active inhibitor to the synthesis of mycotoxins by phytopathogenic fungi: in the presence of arachidonic acid *F. oxysporum* and *F. tricincium* do not synthesize zearalenone, and *P. lilacinum* - roquefortine and fellutanine.

Keywords: Fungus Mortierella Alpina, Arachidonic Acid, Phytopathogenic Fungi, Mycotoxins

1. Introduction

The reduction in the use of toxic chemicals and replacing them with biologically safe products is a key area of research in plant protection in the science world. Biotech companies that implement biological plant protection methods based on the use of bacteria, viruses, entomopathogenic fungi and nematodes are actively developed. In Europe, in 2010 the value of sales of biological products was €24 million [1].

The most promising approach to plant protection against phytopathogens is in the application of microbial metabolites. Specialists have researched bactericidal, fungicidal, nematodocidal, and immunomodulating properties of a number of organic acids: succinic, α-ketoglutaric, citric, arachidonic, palmitoleic acid, and others [2-9].

The role of arachidonic acid (AA) as an inductor (elicitor) to defensive functions in plants has been studied in detail; the application of AA in very low concentrations increases plant resistance to bacterial and fungal pathogens. The immunization by AA is based on the bionic approach – the replication of natural immunity inducers. The immunity of plants, which was acquired under the influence of AA, is non-specific, systemic, as it accelerates healing and increases the plants resistance to stress, in particular, the temperature shocks. Immunization not only increases the yield, but also reduces its contamination by microorganisms, reduces the spoilage during the storage of fruits, root and tuber crops [2, 9].

The practical application of organic acids is limited by the absence of the mentioned compounds of required quality with natural conformeric and isomeric composition. It is known that the presence of even a small amount of impurities in the chemically produced preparations decreases the quality of preparations and makes them toxic for plants. Microbiological production of the mentioned organic acids could be a promising alternative to chemical synthesis. These processes allow researchers to obtain the products of higher quality without harm to soil ecology compounds. They are effective in the economic aspect due to a reduction of cost by around 2-3 times [9].

One of the most common phytopatogenic fungi infected root and tuber crops are fungi belonging to the genera Fusarium and Penicillium; their toxins contaminate grain, silage, and animal feed [10, 11, 12]. The high danger of mycotoxins is that they exert toxic effects in extremely small amounts and are able to intensively diffuse into foodstuffs and cause various diseases that anything from acute skin damage to cancer. The phytopathogenic fungi produce up to 500 low-molecular mycotoxins. As a rule, mycotoxins are secondary metabolites, indole-containing alkaloids [13].

The goal of this work was to determine the effect of the culture medium of *Mortierella alpina* LPM-301 and AA isolated from mycelium in colony formation and the biosynthesis of mycotoxins of phytopathogenic fungi infected alfalfa cultivars.

2. Materials and Methods

All reagents used during this work were purchased from Mosreactiv (Moscow, Russia) and Sigma-Aldrich (St. Louis, MO, USA) and had a highly pure or chemically pure grade.

The phytopathogenic fungi *Purpureocillium lilacinum* F-RKM 0758, *Fusarium tricinctum* F-RKM 0759 and *Fusarium oxysporum* F-RKM 0757 were isolated from the rhizosphere and seeds of alfalfa cultivar Kokorai, growing in Southern Kazakhstan. Phytopathogenic fungi were maintained in test tubes on wort agar slopes.

The cultural medium and biomass enriched by AA was produced with *M. alpina* LPM-301, which was previously selected as an active AA producer [14]. Fungi were grown in 750-ml Erlenmeyer flasks with 100 ml of medium containing (g l^{-1}): glycerol, 73; KNO$_3$, 1.5; KH$_2$PO$_4$, 2.0; MgSO4 ·7H$_2$O, 0.15; CaCl$_2$ ·6H$_2$O, 0.12; yeast extract (Difco, United States), 5.0; trace elements (mg l^{-1}): FeSO$_4$ ·7H$_2$O, 14.9; MnSO$_4$ ·4H$_2$O, 0.2; ZnSO$_4$ ·7H$_2$O, 8.1; CuSO$_4$ ·5H$_2$O, 3.9. Cultivation was performed by a shaker (200 rpm) at 28 ºC for 10 days.

To determine fatty acid composition, mycelium *M. alpina* was vacuum-dried at 70 ºC to a constant weight and subjected to acid methanolysis. Fatty acid methyl esters were analyzed by GLC and identified using standard mixture of fatty acid methyl esters (Serva, Germany). Total lipids were calculated as the sum of fatty acids; heptadecanoic acid was used as an internal standard.

The culture liquid filtrate (without cells) in an amount of 5 ml, 10 ml, and 40 ml was introduced into 200 ml of potato dextrose agar (PDA), which was prepared as follows: 200 grams of sliced peeled potatoes were cooked for 30 minutes in 1 liter of water, further filtered through gauze; water was poured to the filtrate to reach the previous volume, and 20 g of agar and 3 g of glucose were added. PDA with the filtrate was poured into Petri dishes, 20 ml per dish. After solidification, 0.1 ml of suspension of the phytopathogenic fungus was added to the surface of the suspension and ground with a sterile spatula.

To study the effect of AA, 3 ml of preparation was added to 200 ml of PDA (the final AA concentration in the nutrient medium was 0.6 %). The medium was poured into Petri dishes with an added suspension of the phytopathogenic fungus. The number of grown colonies was counted in the test and control groups after 7 days of growth of phytopathogenic fungi at 28 °C.

The effect of AA from the fungus *M. alpina* on the formation of mycotoxins synthesized by the fungi *F. oxysporum, F. tricinctum,* and *P. lilaci* was examined as follows: AA in an amount of 3 ml was added to 200 ml of the Abe's medium with the following composition (g l^{-1}): mannitol - 50; succinic acid - 5.4; MgSO$_4$·7H$_2$O − 0.3; KH$_2$PO$_4$ - 1.0; the pH was adjusted to 5.4 with concentrated NH$_4$OH. 5 ml of a suspension of the examined phytopathogenic fungus were introduced in a flask, and each type of fungus was cultivated for 12 days at 27 ± 1 °C in a shaker (180-200 rpm).

The toxins were extracted from the culture liquid filtrate and mycelium with butanol on the 12th day of growth. The extracts were dried with anhydrous sodium sulfate, filtered, and evaporated to dry in vacuo. The chromatographic analysis of the extracts for toxins produced by phytopathogenic fungi, as well as evaluation of the effect of AA from the fungus *M. alpina* on the changes in the quantitative and qualitative composition of mycotoxins were carried out in a thin layer of chromatography (TLC) which is a universal method for determining all mycotoxin species. The thin layer of chromatography includes the following basic operations: 1) the application of the analyzed sample to the sorbent layer with a capillary; 2) the separation of the sample components into separate zones in the flow of the mobile phase; 3) the detection of zones on the sorbent layer (UV-light, reagent forming colored compounds with separated substances).

As the mobile phase, we used a solvent system chloroform/methanol/ammonia (90:10:0.1). As a sorbent, plates for TLC Silica gel 60 F254 plates (Germany) were used. Samples on the plates were introduced with a capillary in the amount of 1 μl. The substances were detected by UV absorption and fluorescence under UV light and after spraying the plates with Ehrlich's reagent [15]. The results were compared with reference substances.

Toxins contaminating grains, silage, and animal feeds were used as reference substances, including: roquefortine (ML-0406 Sigma); fellutanine, zearalenone (isolated in the Laboratory of Secondary Metabolites at the G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino).

All the data presented are the mean values of three experiments and two measurements for each experiment; standard deviations were calculated (S.D.<10 %).

3. Results and Discussion

3.1. Production of active metabolites by the fungi M. alpina

The technique of active metabolites production has two stages: (1) the fermentation of selected natural strains *M. alpina* LPM-301; and (2) the isolation of the biomass enriched lipids with a high content of AA from the culture liquid. It was found that the biomass of *M. alpina* contained large amount of lipids (27.6 % of dry biomass). Lipids contained saturated and unsaturated fatty acids with the carbon chain length of C$_{14}$ to C$_{22}$. Lipids were represented mainly by AA (40% of lipid). In addition to AA, predominant fatty acids included: palmitic, stearic, oleic, and linoleic acids, which consisted of 10.6; 16.7; 21.1, and 13.7% of lipids, respectively. It was revealed that the studied oleaginous fungal strain excreted organic acids into the culture medium; concentrations of citric, acetic, succinic, and fumaric acids consisted of 0.810, 0.820, 0.246 and 0.24 g/l, respectively.

The culture liquid filtrate that contained active compounds - citric, acetic, succinic, and fumaric acids, and fatty acid methyl esters, extracted from biomass of *M. alpine* were used for further studies.

3.2. Testing of the Colony Formation

Data on the effect of culture liquid filtrate and AA on colony formation by phytopathogenic fungi *P. lilacinu, F. tricinctum* and *F. oxysporum* is shown in Table 1. As seen from this table, the incubation of *P. lilacinu* with 5 ml culture liquid filtrate stimulated the colony formation by 13% and inhibited it by 56% with 40 ml culture liquid filtrate. The incubation of *P. lilacinu* with AA increased the colony formation by 62%.

As seen from table 1, the incubation of *F. tricinctum* with 5 ml culture liquid filtrate inhibited the colony formation between 53% and 96% after incubation with 40 ml culture liquid filtrate. The incubation of *F. tricinctum* with AA reduced colony formation by 69%.

As seen from table 1, the incubation of *F. oxysporum* cells with 5 ml culture liquid filtrate did not restrict cell growth, while the increase in the concentration of filtrate to 40 ml inhibited the colony formation by 84%. The incubation of *F. oxysporum* with AA resulted in the inhibition of colony formation by 90%.

Thus, the inhibiting effect of culture liquid filtrate containing active compounds - citric, acetic, succinic, and fumaric acids and AA on pathogenic fungi that cause plant diseases were revealed in the present study.

It should be mentioned that in recent years more and more data has been published on the antimicrobial potential of pure organic acids including citric acid, succinic acid, α-ketoglutaric acid, and palmitoleic acid. Citric acid inhibits the growth of phytopathogenic fungus Colletotrichum, which causes anthracnose (cucumber and melon disease) [3]. Succinic acid produced by microbiological synthesis and inhibited the growth of bacterium *Erwinia carotovora* and fungus *Penicillium casei* causing black leg (soft rot) and blue rot (penicilliosis) of potato, respectively [6]. α-Ketoglutaric acid suppressed the growth of phytopathogenic fungus *Fusarium napiforme* and nematodes *D. destructor* [7]. It revealed a high antimicrobial potential of palmitoleic acid. It was found that ethyl- and methyl esters of palmitoleic acid possess high inhibitory activities that can be used against such pathogens as *Streptococcus mutans*, *Candida albicans*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis* [4].

It should be noted that there is only scarce literature data concerning the antifungal potential of the culture liquid filtrates of

bacteria and fungi. Todorova and Kozhuharova [16] reported that *Bacillus subtilis* culture liquid filtrate had a high inhibitory activity against the fungi *Alternaria solani*, *Botrytis cinerea*, *Monilia linhartiana*, *Phytophthora cryptogea*, and *Rhizoctonia* sp. Yoshida *et al.* [17] reported that *B. amyloliquefaciens* RC-2 culture liquid filtrate isolated from mulberry leaves also prevented the growth of phytopathogenic fungi and bacteria such as *Rosellinia necatrix*, *Pyricularia oryzae*, *Agrobacterium tumefaciens*, and *Xanthomonas campestris pv. campestris*. Shemshura et al. [8] showed that the culture of liquid filtrate from the fungus *Aspergillus candidus*, comprising citric acid and 1,2-dimethyl citrate, exhibited nematicidal activity against parasitic nematodes *Ditylenchus destructor* being especially dangerous for potatoes, and root-knot nematodes *Meloidogyne incognita*.

3.3. Testing of the Biosynthesis of Mycotoxins

To establish the mechanism of the AA inhibitory effect on the phytopathogenic fungi *F. oxysporum, F. tricinctum*, and *P. lilacinum*, affecting alfalfa, a chromatographic analysis has been carried out for the extracts from the mycelium and culture liquid of the fungi, which were obtained by culturing them in the presence of AA (test group) and in the absence of AA (control group).

We used a thin layer of chromatography for analysis of mycotoxins isolated from *P. lilacinum*, *F. tricinctum* and *F. oxysporum*. This method allowed us to identify metabolites based on the chromatographic mobility (Rf), fluorescence, and color reaction within specific reagents in the presence of reference substances [15].

We used the most common toxins synthesized by fungi of the genus Fusarium as reference substances (standards): zearalenone (Rf=0.02, a sky-blue glow under UV light and a brown-blue color after treatment with Ehrlich's reagent); fellutanine A (Rf=0.14, a turquoise glow under UV light and a violet-blue color after treatment with Ehrlich's reagent) and toxin synthesized by fungi of the genus Penicillium: roquefortine (Rf=0.30, yellow fluorescence under UV light, and a blue color after treatment with Ehrlich's reagent).

Table 2 presents data on the effect of AA in the qualitative and quantitative compositions of mycotoxins present in the mycelium extracts from *F. oxysporum, F.tricinctum* and *P. lilacinum*. As seen in this table, the mycelium extract of *F. oxysporum* are grown on the AA-free medium (control group), 10 metabolites were detected, and only 6 of them were attributed to the substances of indole nature. At the same time, the total amount of metabolites in the mycelium extract of the fungus *E. oxysporum*, grown on the medium with AA (experiment), decreased over three times.

As seen in Table 2, the chromatographic results showed the presence of seven metabolites in the mycelium extract of the fungus *F. tricinctum*, grown on AA-free medium (control group), of which five were of indole nature. At the same time, in the AA presence (test group), the total amount of metabolites in the mycelium extract of *F. tricinctum* decreased by more than half, and the amount of indole-containing metabolites decreased from five to one.

As can be seen in Table 2, the mycelium extract of the fungus *P. lilacinum* grown on AA-free medium (control), six metabolites were detected, five of which were of indole nature. At the same time, in the presence of AA (test group) the total number of metabolites in the mycelium extract of *P. lilacinum* decreased twice, three metabolites belonging to alkaloids were dentified: the metabolites with Rf=0.34, Rf=0.44 and Rf=0.93.

Table 3 presents data on the effect of AA from the fungus *M. alpine* on the qualitative and quantitative composition of mycotoxins present in the culture liquid extract of the examined phytopathogenic fungi. As seen from Table 3, nine metabolites were detected in the culture liquid extract of *F. oxysporum*, of which seven gave a positive reaction with Ehrlich's reagent. At the same time, in the presence of AA (test group) the total amount of

metabolites in the culture liquid extract of *F. oxysporum* decreased four and a half times, six of indole substances were not synthesized, and one indole-containing metabolite with Rf=0.24 was detected in the component composition.

Chromatographic data for the culture liquid extract of the fungus *F. tricinctum* revealed the presence of 10 metabolites that gave a positive reaction with Ehrlich's reagent. At the same time, in the presence of AA (test group), there was a fiv-fold decrease in the number of indole-containing metabolites in the extract of *F. tricinctum* culture liquid; only two metabolites were detected: a metabolite with Rf=0.68 and a metabolite with Rf=0.85.

As seen from Table 3, when cultivating the fungus *P. lilacinum* in the AA-free medium (control group), nine metabolites were detected in the culture liquid extract, of which five belonged to indole-containing alkaloids. Alongside with that, in the presence of AA (test group) the total amount of metabolites in the culture fluid extract of the fungus *P. lilacinum* decreased 2.25 times, and alkaloids with detections of Rf=0.20 and Rf=0.85.

By analyzing the data and evaluating the effect of the AA preparation on the toxin of biosynthesis in phytopathogenic fungi *F. oxysporum, F. tricinctum, and P. lilacinum*, we have found that when phytopathogenic fungi are grown on a medium with AA, they do not produce a number of mycotoxins. When the fungus *F. oxysporum* was grown in the AA-free medium, six metabolites were detected in the mycelium extract, and seven metabolites belonging to indole-containing alkaloids were found in the culture liquid. At the same time, the number of toxins decreased to one both in the extracts from both the mycelium and culture liquid in the presence of AA (Tables 2, 3). The metabolites found in the control variants of the mycelium extract with R_f=0.02 and culture liquid extract with R_f=0.03, which gave the sky-blue fluorescence and brown-blue color with the Ehrlich's reagent, corresponded to the same parameters of the mycotoxin zearalenone (reference standard). In the experimental variants (supplemented with AA), the formation of this toxin by the fungus *F. oxysporum* was not confirmed both in the mycelium and in culture liquid (Tables 2, 3). When the strain *F. tricinctum* and the fungus *F. oxysporum* were grown in the medium with AA, a decrease was recorded in the amount of toxins produced, including the metabolite present in extracts from the mycelium and culture liquid corresponding to zearalenone (reference standard) (Tables 2,3).

A number of authors who have noted that zearalenone is one of the most common toxins synthesized by fungi of the genus *Fusarium*, which contaminate grains, silage, and animal feeds [12]. The effect of the AA preparation on reducing the formation of toxic substances synthesized by fungi of the genus *Fusarium*, including zearalenone, makes it promising as a fungicide for protecting fodder crops from contamination by toxin-producing fungi.

A comparative chromatographic analysis of extracts from the mycelium and culture liquid of the fungus *P. lilacinum* revealed the presence of 5 alkaloids in the mycelium and culture liquid. Metabolites with R_f=0.15 (mycelium extract) and R_f=0.14 (culture liquid extract) by their chromatographic mobility, a turquoise glow under UV light, and violet-blue color after treatment with Ehrlich's reagent corresponded to fellutanine used as a reference standard. In addition to fellutanine, a metabolite with R_f=0.31, having a yellow glow under UV light and a blue color after treatment with Ehrlich's reagent, was detected in the culture liquid of the fungus *P. lilacinum*, which corresponded to roquefortin used as a reference standard (Tables 2,3). The synthesis of mycotoxins felutanine and roquefortin in fungi of the genus *Penicillium* was described in the literature; the chromatographic characteristics of metabolites which we obtained when examining extracts from the mycelium and culture liquid of fungus *P. lilaci* corresponded to the literature data (Zelenkova et al. 2003). It was noted that in the extracts from both the mycelium and culture liquid of the fungus *P. lilacinum*, grown on a medium supplemented with AA (test group), the total number of metabolites decreased by two times, and the number of toxins

representing indole-containing alkaloids was reduced from five metabolites to one. In this case, toxins corresponding to fellutanine and roquefortin used as reference standards, were absent (Tables 2, 3). The synthesis of fellutanine A in fungi of the genus *Penicillium* was described in the literature. Our data on the chromatographic mobility, UV glow, and color development after treatment with Erlich's reagent are consistent with the results obtained by Zelenkova et al. [15].

4. Conclusion

Thus, the inhibiting effect of culture liquid filtrate *M. alpina* containing active compounds - citric, acetic, succinic, and fumaric acids and AA on pathogenic fungi that cause plant diseases were revealed in the present study.   It has been established that the phytopathogenic fungi, grown in the presence of arachidonic acid, do not produced several mycotoxins, in particular, F. oxysporum and F. tricincium do not synthesize zearalenone, while the fungus P. lilacinum - roquefortine and fellutanine. The preparation from the fungus *M. alpina* can be used in the future as a basis for creating an environmentally friendly fungicide to protect forage crops from damage by toxin-forming fungi.

Acknowledgement

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