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Primary analysis of new measures against fungal diseases of woody plants

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The aim of this work was to determine the impact of bacterial isolates Tx and Ux (of two kinds) from spontaneous fruit-berry fermentation upon fungal disease agents from the genera *Alternaria* and *Fusarium*. The disease agents were isolated from various ornamental plants growing in the city greenery. The killer activity of bacterial isolates is determined by the ability of the test strains to form lysis zones on the lawns of the test α' *S. cerevisiae* strain and plant disease agents. *S. cerevisiae* standard K7, Rom-K100, M437, MS300 killer strains were used as a control. It has been previously demonstrated that the toxins produced by Tx and Ux bacterial isolates are able to destroy not only yeasts of the genus *Saccharomyces* but also of the genera *Candida*, *Kluyveromyces* as well as such plant disease agents as *Verticillum albo-atrum* and *Venturia ineaqualis*. Tests of the impact of these toxins upon fungi of the genera *Alternaria* and *Fusarium* revealed the highest killing activity during the intensive growing stage on the YEPD and MB media (pH 4.8) at a temperature of 20–30 °C. The obtained results could be employed while elaborating new and efficient plant protection measures.

Key words: killer effect, bacteria, yeasts, micromycetes, fungal diseases

INTRODUCTION

A search for new biologically active substances characterized by antimicrobial activity against many bacterial and yeast pathogens is particularly relevant for all pathology specialists [1]. Recently, antibiotic properties of killer toxins produced by yeasts have been intensively studied together with the possibilities to apply them for antifungal immunotherapy [2]. A toxin, attacking the targets on the surface of microorganism or yeast cells, destroys sensitive cells but has no toxic effect upon cells of higher eukaryotes. This property is significant not only in medicine, but also for creating new phytopathogen-resistant plant cultivars. Therefore, the search for microorganisms producing toxins of a wide activity spectrum and characterized by killer antipathogen properties is in progress, aimed to contribute to solving the problems related with plant protection [3]. It is especially important for the management of greenery in cities, because due to various biotic and abiotic factors weakened plants are infected by fungal diseases and pests [4]. Spores of the parasitic *Alternaria, Cytospora, Fusarium*, *Nectria*, *Phomopsis* fungi block water vessels, cause the drying of the above-ground parts of plants [5, 6], leaf spots; thus, plants loose their ornamental value [7, 8].

To protect both ornamental and crop plants, various measures – agrotechnical, physical-mechanical, biological, quarantine and chemical – are applied. Their goal is to reduce the number of plant pests and disease agents. None of these measures is universal for all plant pests and disease agents, so they are applied systemically in order to get the best possible result. The physical-mechanical method is applied in city greeneries during selective sanitary cuttings [9, 10]. The phenomenon of mycorrhiza is mentioned by many authors as the method of biological control. Chemical measures are applied most extensively as their impact upon pests is faster than of other protection measures [11, 12]. In ornamental greeneries, however, chemical control substances are not widely applied due to a possible harm to the health of people and animals. Therefore, it is essential to search for new efficient protection measures. One of such ways could be the application of new killer yeasts and other microorganisms that counteract the plant disease agents.

During earlier investigations, the new toxin-producing bacterial isolates Tx and Ux characterized by killer impact upon certain micromycetes, including plant disease agents *Venturia inaequalis* and *Verticillium albo-atrum,* were tested. They had been isolated from spontaneous fruit-berry fermentations employing multiple cloning, so probably they could be safe to people and animals (further investigations are required) [13].

The aim of this work was to test the impact of their toxins upon the plant disease agents ascribed to the genera *Alternaria* and *Fusarium*.

According to the reference data, the search for new killer yeasts and micromycetes characterized by antipathogenic

properties is highly relevant; their biochemical and genetic analyses are highly promising. Every year new microorganisms possessing antipathogenic features are revealed [14].

MATERIALS AND METHODS

Isolation of micromycetes from ornamental plants. A nutritious medium – malt extract agar (MEA) pH 4.8 – was used for the isolation of micromycetes. Pieces of dry branches were placed on the agar medium into each Petri dish. Till the appearance of fungal mycelium, the closed dishes were incubated in a thermostat at a temperature of 24 °C [15]. The culture was purified employing cloning and microscopy. Later the micromycete colonies were transferred into separate Petri dishes with MEA medium. The following pure cultures were obtained: *Alternaria alternata* (Fr.) Keissl., *Alternaria* sp., *Fusarium culmorum* (W.G.Sm.) Sacc., *F. graminearum* Schwabe, *F. sambucinum* Fuck., *F. semitectum* Berk. & Ravenel, *F. solani* (Mart.) Sacc., *F. sporotrichioides* Sherb., *F. oxysporum* var. *orthoceras* (Appel & Wollenw.), *Phomopsis irregularis* (Died.) Petr., *Cytospora* sp. [16, 17].

The pathogen species were identified basing on macro- and micromorphological properties (colony colour, shape, growth rate, mycelium and spore size, colour, form). Micromycete species were identified according to various manuals and reference books [18, 19].

Determination of killer activity. Killer activity of Tx and Ux bacterial isolates is determined by their ability to form lysis zones on lawns of the test strains. The *S. cerevisiae* strain α'1 (MATα, *leu2-2 [kil-0]*), sensitive to all killers, was used for testing the activity of killer toxin. *S. cerevisiae* killer strains K7 (MAT*a*, *arg9 [kil-K1]*), Rom-K100 (wt, *HM/HM [kil- K2]*), M437 (wt, *HM/HM [kil-K2]*), MS300 (MATα *leu2 ura3- 52 [kil-K28]*) were employed for the control [20].

The bacterial isolates T1x, T2x, T3x, Ux have been obtained by spontaneous fermentation of fruits and different berries. Yeast cells were grown in YEPD medium containing 1% of yeast extract, 2% of peptone and 2% of glucose. Buffered methylene blue medium containing YEPD adjusted to the required pH using 0.2 mol/l citrate–phosphate buffer and 2% of agar was used for the killer activity and immunity test (medium MB). It was also used for testing the killer phenotype (pH 4.8). At such pH level the action of the control killer strains of *S. cerevisiae* is clearly observable, and the chosen pathogens grow well of this medium [21].

The killer phenomenon was tested by sowing the sensitive α'1 strain into the medium applying the deep sowing method; the test and standard strains were sown on the surface of the formed lawn. The medium was spread in a thin layer into Petri dishes. The dishes were dried through the night at room temperature. 10 ml of melted medium cooled to 45 °C was supplemented with a suspension of the test yeast cells up to 10^5 cell/μl. The medium was poured over the prepared dishes with a bottom layer of agar. As the upper agar layer gelatinized, the colonies of the test cultures were sown with a tag. The dishes were incubated for three days at 24 °C. Around the colonies producing killer toxin, a lawn of a strain sensitive to this toxin does not grow, therefore clear lysis zones are formed. The activity of the produced toxin was quantitatively evaluated using the method proposed by Gulbinienė et al. [22].

The plant disease agents were grown in two ways: sown by the surface method on YEPD and MB media or by the deep sowing method by suspending in sterile water and mixing with melted and cooled to 35 °C MB and YEPD media with the further spreading of the suspensions in Petri dishes. As the disease agents were sown on both media by both deep and surface methods (similarly as control strains of *S. cerevisiae*), the toxin-producing bacterial isolates Tx and Ux were transferred immediately or after the appearance of fungal mycelium (after two days).

RESULTS AND DISCUSSION

The killer effect of bacterial isolates (marked Tx and Ux) was tested against the following fungal disease agents of ornamental plants: *Alternaria alternata* (Fr.) Keissl., *Alternaria* sp., *Fusarium culmorum* (W.G.Sm.) Sacc., *F. graminearum* Schwabe, *F. sambucinum* Fuck., *F. semitectum* Berk. & Ravenel, *F. solani* (Mart.) Sacc., *F. sporotrichioides* Sherb., *F. oxysporum* var. *orthoceras* (Appel & Wollenw.). Samples of fungal disease agents were gathered in the streets, parks, and squares of Vilnius from various woody plants: linden (*Tilia* L.), Norway maple (*Acer platanoides* L.), horse-chestnut (*Aesculus* L.), poplar (*Populus* L.), etc.

Previous investigations revealed that on the MB medium toxins of the bacterial isolates Tx and Ux were killing lawns of a sensitive *S. cerevisiae* α'1 strain. In case of Tx, the lysis zones up to 15 mm and in case of Ux up to 20–25 mm were recorded. *S. cerevisiae* standard K7, K100, M437, MS300 killer strains were used as a control; the lysis zones of the excreted toxins were 8–15 mm in diameter (Fig. 1).

It has been previously demonstrated that the revealed microorganisms are able to destroy not only yeasts of the genus *Saccharomyces*, but also of the genera *Candida*, *Kluyveromyces* as well as phytopathogens *Verticillum albo-atrum* and *Venturia ineaqualis*; therefore, their ability to influence other plant disease agents was tested as well.

The impact of toxins on *Alternaria* and *Fusarium* fungi, disease agents of some woody plants, was tested. First of all it has been determined that the test plant pathogens grow on the MB and YEPD media at 24–30 °C. On these media, bacterial isolates Tx and Ux were producing toxins and killing the lawns of a sensitive *S. cerevisia* α'1 strain. Besides, it has been determined that bacterial isolate Tx grows well and produces toxins on the above-mentioned media at a temperature of 20–37 °C. Ux also intensively produces toxin and destroys lawns of the sensitive *S. cerevisia* α'1 strain on

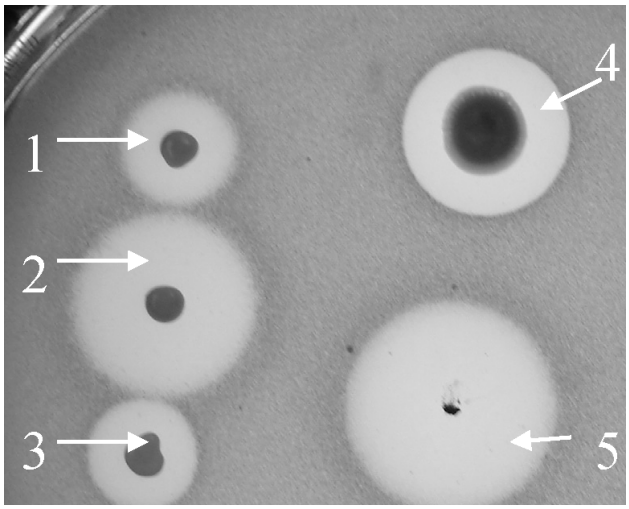
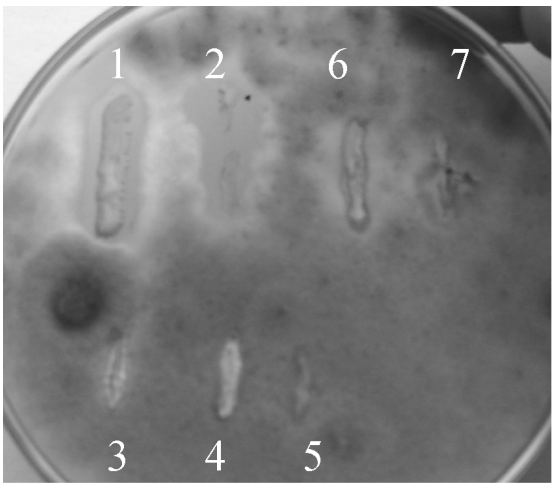
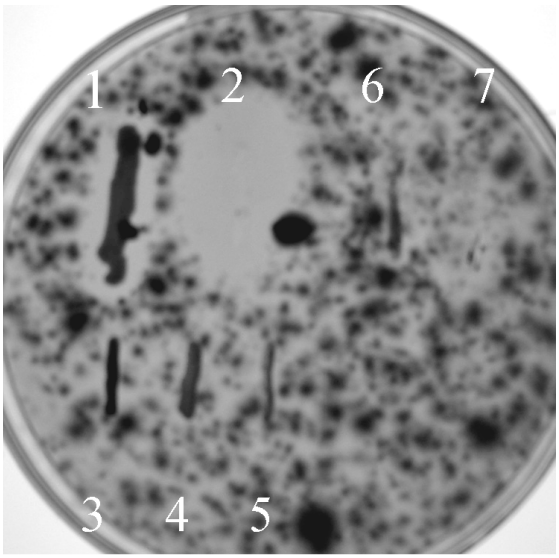


Fig. 1. Comparison of the impact of *Saccharomyces cerevisiae* killer strains and Ux and Tx upon a sensitive α' strain: *1* – Rom-K100, *2* – M437, *3* – K7, *4* – Tx, *5* – Ux



A



B

Fig. 2. MB medium, pH 4.8. *Alternaria* sp. A – surface lawn, B – deep lawn. *1* – Tx, *2* – Ux, *3* – K7, *4* – M437, *5* – MS 300 (contact inhibition) transferred immediately, *6* – Tx, *7* – Ux (transferred after 2 days)

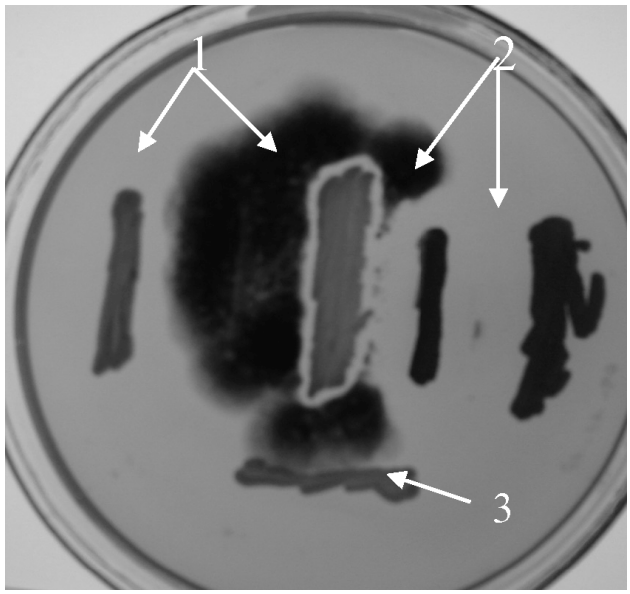


Fig. 3. YEPD medium. Inhibition of *Alternaria* sp. growth. *1* – Tx (small lysis zone), *2* – Ux (very strong impact), *3* – M437 (transferred immediately). Only contact inhibition

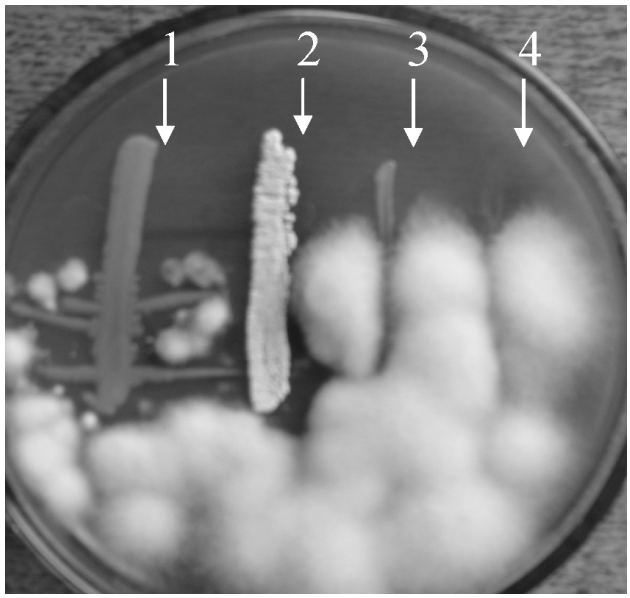


Fig. 4. YEPD medium, *Fusarium* sp. surface sowing: *1* – Tx, *2* – Ux (all cultures transferred simultaneously), *3* – Tx, *4* – Ux (transferred after 2 days)

these media at a temperature of 20–37 °C, but the optimal growth temperature is 30 °C.

Therefore, their toxins are characterized by wide activity spectra in both pH (pH 4–7) and temperature (20–37 °C) intervals.

The toxins affected *Alternaria* sp. and *Fusarium* sp. pathogens when deep sowing and surface sowing methods were applied on the MB medium (Fig. 2 A, B).

The toxin-producing bacterial isolates Tx and Ux were grown in liquid media. Sterile filtrates of Ux and Tx toxins were tested on *Alternaria* and *Fusarium* strains as well as on the control strains of *S. cerevisiae*. 100 µl of toxin filtrate formed on them standard, completely clear lysis zones. Meanwhile on the test pathogens their im-

pact was evident only at the beginning of incubation; at the end of incubation the fungal mycelium neutralized the effect, showing that the toxins disintegrate because the cultivation interval is too long. A permanent impact of an active toxin is needed, i. e. immediate inoculation of Tx and Ux produces the largest lysis zones. When Tx and Ux were transferred on the medium after two days, considerably smaller lysis zones formed. After inoculation of the cultures, toxins are constantly produced; therefore, the lysis zones persist for a long time. The control killer strains of *S. cerevisiae* on the MB medium were characterized only by contact inhibition. On the YEPD medium their toxins were not active, because they did not fit into the pH interval of their activity. The edges of *Alternaria* sp. and *Fusarium* sp. lysis zones are not very clear-cut. It can be explained by the specificity of fungal growth, the variation of growth rates and consumption of the substances in the medium. As substances in the medium are consumed, in case of the incubation up to 20 days, the fungus slowly diminishes the lysis zones.

As can be seen from the figures, the toxin produced by Ux isolate forms the largest lysis zones. Tx, however, is characterized by a wider temperature interval suitable for growth and grows better on both media. Ux grows best on the YEPD medium, while on MB it grows poorly but produces toxin rather intensively (Figs. 3, 4).

For further research, biochemical and genetic nature of the toxins should be investigated; much more other plant pathogens should be tested. It is also necessary to try cloning the genes that could be used for creation of genetically modified organisms.

Our research as well as previous investigations of other specialists demonstrate that the toxins produced by the bacterial isolates Tx and Ux could affect many more microorganism species, i. e. they have a rather wide activity spectrum and may be very promising for both scientific research (killer and immunity phenomena) and practical application.

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NAUJŲ APSAUGOS PRIEMONIŲ PRIEŠ SUMEDĖJUSIŲ AUGALŲ GRYBINIŲ LIGŲ SUKĖLĖJUS PIRMINĖ ANALIZĖ

Santrauka

Šio darbo tikslas yra nustatyti bakterijų izoliatų, išskirtų iš vaisių ir uogų spontaninių raugų bei pažymėtų Tx ir Ux (dviejų rūšių), poveikį augalų grybinių ligų sukėlėjams *Alternaria* ir *Fusarium* genčių. Ligų sukėlėjai buvo išskirti iš įvairių dekoratyvinių augalų, augančių miestų želdynuose. Tx ir Ux bakterijų izoliatų žudantis aktyvumas nustatomas pagal testuojamų kamienų gebėjimą suformuoti lizės zonas ant testerinio α' *S. cerevisiae* kamieno ir augalų ligų sukėlėjų gazonų. Kontrolei naudoti *S. cerevisiae* standartiniai K7, Rom-K100, M437, MS300 kamienai-žudikai. Anksčiau buvo nustatyta, kad Tx ir Ux bakterijų izoliatų produkuojami toksinai gali sunaikinti ne tik *Saccharomyces, Candida, Kluyveromyces* gentims priklausančias mieles, bet ir kai kuriuos augalų ligų sukėlėjus – obelinį rauplėgrybį (*Venturia inaequalis*) ir balzganąjį menturgrybį (*Verticillium albo-atrum*). Patikrinus šių toksinų poveikį *Alternaria* ir *Fusarium* genčių grybams, paaiškėjo, kad jie geriausiai žudo minėtus grybus intensyvaus augimo fazėje ant YEPD ir MB terpių, kai pH 4,8, temperatūra 20–30 °C. Gauti tyrimų duomenys gali būti panaudoti kuriant naujas efektyvias augalų apsaugos priemones ir prieš minėtų genčių grybus.