Primary analysis of new measures against fungal diseases of woody plants

V. Meškauskienė¹, V. Melvydas²

¹ Laboratory of Phytopathogenic Microorganisms, Institute of Botany, Žaliųjų ežerų 49, LT-08406 Vilnius, Lithuania
² Laboratory of Genetics, Institute of Botany, Žaliųjų ežerų 49, LT-08406 Vilnius, Lithuania
E-mail: genetika@botanika.lt

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 Verticillium albo-atrum and Venturia inaequalis. 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 antipathogenic 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 greenery 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 (MATa, *leu2-2* [kil-0]), sensitive to all killers, was used for testing the activity of killer toxin. *S. cerevisiae* killer strains K7 (MATa, *arg9* [kil-0]), Rom-K100 (wt, *HM/HM* [kil-0]), M437 (wt, *HM/HM* [kil-0]), MS300 (MATa *leu2 ura3-52* [kil-K2]) 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 10⁶ 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.), 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 *Verticillium albo-atrum* and *Venturia inaequalis*; 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. cerevisiae* 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. cerevisiae* α'1 strain on
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 inocula-
tion of the cultures, toxins are constantly produced; the-
therefore, the lysis zones persist for a long time. The con-

trol 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 con-
sumption 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 po-

orly 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 phenome-


7. Snieškienė V, Juronis V. Bulletin of Polish Academy of
8. Butin H, Kehr R. Nachrichtenbl Deut Pflanzenschutz 1999;
10. Juronis V, Snieškienė V. Baltic Botanic Gardens 2003:
76–9.
11. Juronis V, Snieškienė V. Urban Forestry in Nordic and
12. Butin H. Tree Diseases. Causes, Biology and Control in
Genetical and Physiological Fundamentals of Plant Growth
15. Arx JA. The Genera of Fungi Sporulating in Pure Cul-
17. Turner AS, Lees AK, Rezanoor NH, Nicholson P. C.
18. Nelson PE, Toussoun TA, Marasas WFO.: Fuscariun Spec-
es: an Illustrated Manual for Identification. Pennsylva-
19. Ellis MB. More Dematiaceous Hyphomycetes. CMI, Kew:
20. Kondratienė L, Gulbinienė G, Servienė E, Melvydas V.

V. Meškauskienė, V. Melvydas

NAUJŲ APSAUGOS PRIEMONIŲ PRIEŠ SUMEDĖJUSIŲ AUGALŲ GRYBINIŲ LIGŲ SUKELĖJUS PIRMINĖ ANALIZĖ

Santrauka
Šio darbo tikslas yra nustatyti bakterijų izoliatų, išskirtų iš vaisių ir uogų spontanių raugių bei pažįstų Tx ir Ux (dviejų rūšių), poveikį augalų grybinių ligų sukeltiems Alternaria ir Fusarium genčių. Ligų sukėlėjai buvo išskirti iš įvairių dekoratyvinių auga-

lių, augančių miestų žydyniuose. Tx ir Ux bakterijų izoliatų įždan-
tis aktyvumas nustatomas pagal testuojamų kaimiškų gebėjimą su-
formuoti žiūnas ant testuojamų grybų. Patikrinus S. cerevisiae
standartinius K7, Rom-K100 ir M437, MS300 kaimiškas ūkis. Arksčiausiu buvo nustatyta, kad Tx ir Ux bakterijų izoliatų produkuoju tok-

sinau susirinkti ne tik Saccharomyces, Candida, Kluyveromy-
ces gentis priklausančias mieles, bet ir kai kurios augalų ligų
sukėlėjus – obelinų rauplėrygį (Venturia inaequalis) ir balzgan-

jąjį mėntargrygį (Verticillium albo-atrum). Patikrinus šį tokius
po-veikį Alternaria ir Fusarium genčių grybams, paaiškėjo, kad jie
geriausiai žudo minėtus gyvus intensyvaus augimo faze ir net po
eyPD ir MB terpių, kai pH 4,8, temperatūra 20–30 °C. Gauti ty-

rimų duomenys gali būti panaudoti kuriant naujas efektyvias
augalų apsaugos priemones ir priež minėtų genčių gyvbus.