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# Tartaric acid synthetic derivatives effect on phytopathogenic bacteria

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**Abstract.** The scientific goals of current research were devoted to targeted derivatization of natural tartaric acid (TA) for the enhancement of antimicrobial properties of it such as like the effects of them on multi-drug resistant phytopathogenic bacteria, depends to their structure features and the genetic parameters of studied microorganisms. The main utilitarian goal is to develop new class of antimicrobial biodegradable compounds with possible prospective application as moresafe alternative to traditional antibiotics applicable for: agriculture, horticulture, food industry as well as in medicine. These compounds were developed in basic research laboratory: 'Agrarian Pesticide Creation and The Quality Control' at National Polytechnic University of Armenia (NPUA). TA and tartrates are safe antimicrobial food additives. According to the results of *in vitro* studies, the synthesized cyclohexyl-, benzyl- and phenyl- derivatives of it in the form of amine complex salts (correspondently CAS, BAS and PhAS) and cyclic imides (correspondently CI, BI and PhI) are effective against the model multi-drug resistant strains of Gram-negative microorganisms. Bactericidal effects of TA derivatives were demonstrated on 19 model native soil strains of phytopathogenic *Xanthomonas beticola* (*6 strains*), *X. vesicatoria and Pseudomonas syringae* (*13 strains*) representatives, which are differing in antibiotic resistance. Regarding the transformation results, the absence of transfer of resistance to TA imides and amine complex salts by plasmids, makes them promising objects for further research. Primary studies have not shown any antibacterial effect on various non-pathogenic soil bacteria (*Pseudomonas chlororaphis, P. taetrolens*, etc.). The described compounds are recommended for further more detailed toxicological studies.

**Key words:** antimicrobial agents, complex salts of tartaric acid, imides of tartaric acid, multi-drug resistance, phytopathogen, *Pseudomonas*, *Xanthomonas.*

## INTRODUCTION

Phytopathogenic bacteria from *Pseudomonadaceae* and *Xanthomonadaceae* families, which are the causative agents of plant bacteriosis (fruits, leaves, etc.) are very

significant. These microorganisms are ubiquitous on the planet due to their high adaptability and plasticity of metabolism (Yoshimoto et al., 2010).

High adaptability and antibiotic resistance, especially pan-drug resistance and multi-drug resistance of *Pseudomonas* and *Xanthomonas* representatives are well known (*Pseudomonas syringae, pv. syringae, P. syringae, pv. tabaci, P. syringae, pv. lachrymans, Xanthomonas vesicatoria, X. beticola*) (Santi et al., 2021). Their representatives are common inhabitant of almost any wet surface, being harmful for crops, agricultural animals (Morris et al., 2007). In this regard, these bacteria are a convenient object for studying the mechanisms of resistance and ways to overcome it.

The problem of antimicrobial drugs resistance has become particularly acute in recent decades. According to WHO reports for 2019, antimicrobial drugs resistance is one of the top five research priorities for the XXI century (World Health Organization, 2019). Due to the global coronavirus pandemic that has swept the planet, the use of antibiotics and other antimicrobial drugs has increased significantly. It has led to an increase in the number of resistant bacteria (Livermore, 2021). Therefore, the development of new classes of antimicrobial drugs is especially acute (Rahman & Das, 2021). However, promising synthetic antibiotics, such as azalide macrolides or fluoroquinolones, to which no natural specific resistance has been found, are often quite non-selective in their effect. Also, they have a number of undesirable side effects for patients. For example, vancomycin and the analogous antibiotics, used against super bacteria are often cardiotoxic and neurotoxic (Martinez et al., 2018).

Various types of cross-resistance, such as pan-drug resistance, as well as multi-drug resistance in case of phytopathogenic representatives of *Pseudomonadaceae* and *Xanthomonadaceae,* lead to a low efficacy of agricultural crop protection agents containing antibiotics. This problem is well-known also in scope of medicine and veterinary. It's notable by the decrease of antibiotic therapy efficiency of *Pseudomonas, Stenotrophomonas* and *Xanthomonas* infections treatment of animals and plants. That appropriately is caused by the abilities of mentioned bacteria to transmit the resistance by the intraspecific gene horizontal transfer. In contribution to it the enhancement of properties resistance take place, with the uncontrolled spread of antibiotics resistance genes by mobile genetic elements (transposons and plasmids) of phytopathogenic strains and agricultural animal opportunistic pathogens, as well as human pathogens.

As a result, it led to decrease of efficiency of therapy for plant, human and animal *Pseudomonas, Stenotrophomonas* and *Xanthomonas* infections treatment (Monteil et. al., 2016). The negative impact of this problem on health care, gardening, horticulture, agriculture and animal husbandry is quite significant (Vidaver, 2002).

Being involved in a huge number of food chains of various biogeocenoses and other ecosystems, as decomposers, *Pseudomonadaceae* and *Xanthomonadaceae* representatives participate in interspecific gene horizontal transfer. And it led to spread of antibiotics resistance genes by mobile genetic elements (transposons and plasmids). Thus, the number of resistant pathogens of agricultural crops is increasing. In response to this, the volumes of the used antimicrobial plant protection agents are being increased, what adversely affects the quality of agricultural production. It transfers the problem of multi-drug resistance of microorganisms to the plane of agriculture, ecology, and health care (Dolejska & Literak, 2019). The changes of resistance profiles of microorganisms can potentially lead to dramatic changes in animals and plants biodiversity in biogeocenoses and other ecosystems (Aslam et al., 2018). Thus, the main research

problem is the elaboration of compounds which can be more effective and less harmful for human organism and for environment.

That is why, the search for new alternatives to classical, complex and combined agents of the protection of agricultural plants and animals, containing antibiotics of various classes is a very urgent theoretical and practical problem (Granados-Chinchilla & Rodríguez, 2017).

For this reason, one of the most promising directions is the targeted derivatization of natural antimicrobial compounds with the potential enhancement of antimicrobial properties. One of the alternatives for solving of this problem is the use of natural organic acids and their derivatives (Melaku et al., 2021). One of the common groups of antimicrobial compounds are natural organic acids, especially aldaric acids (Liang et al., 2021). These compounds are widespread in plants and constitute one of the most important components of the antimicrobial barrier of human skin (Cartron et al., 2014). As it is known from literature data TA, lactic and citric acids, as well as their derivatives, have proven themselves as effective antimicrobials. Dicarboxylic and tricarboxylic acids and their salts, have shown in many experiments an unexpected ability to enhance the antimicrobial activity of a number of disinfectants. One per cent of citrate content can significantly increase the bactericidal and bacteriostatic activity of antibiotics. It also decreases the viability of strains, which are resistant to them. Citrates have intrinsic antimicrobial properties and they are synergistic effective against bacterial colonization of tissue samples by pathogens. They also enhance the antimicrobial properties of phytogenic polyphenols (activators of the innate immune systems of plants), and disinfecting organic dyes (crystal violet, methylene blue) (Andrews et al., 2011).

Another direction of search for new antimicrobial drugs is the development of polymeric, polyamide and nanopolymeric derivatives of carboxylic acids. Natural aldaric acids derivatives creation by the targeted derivatization and functionalization are the most important directions for development of new classes of antimicrobial agents. The advantage of this method consists in obtaining compounds with alternative mechanisms of activity, which are sharply different from the ways of e of known antibiotics (D'Mello, 2003; Kumari & Jaisankar, 2017).

The principal scientific goal of current research was natural TA targeted derivatization for the increase in antimicrobial potential of it. The main practical goal of a particular study was an application of the elaborated compounds, as new potentially ecologically safe alternative of generally used antibiotics, which are not always effective and moreover often have harmful side effects. Due to well-known safety and antimicrobial properties of TA and its salts, amine complex salts and cyclic imides with improved antimicrobial properties against multi-drug resistant pathogens were synthesized. The effect of cyclohexyl-, phenyl-, benzyl-substituted imides and complex salts of natural TA on strains of native phytopathogenic *Xanthomonas* 13 strains of different species, isolated form soil, was considered (Fig. 1) (Bagdasaryan et al., 2020).

Due to well-known antimicrobial properties of TA and its salts, this paper considers the possibility of the successful use of TA synthetic derivatives: amine complex salts and cyclic imides with improved antimicrobial properties against multi-drug resistant pathogens. The effect of cyclohexyl-, phenyl-, benzyl-substituted imides and complex salts of natural TA on strains of phytopathogenic bacteria various types was considered (Fig. 1) (Bagdasaryan et al., 2020). According to the earlier studies, cyclohexyl- and benzyl-substituted imides and amine complex salts demonstrated activity against

*K. pneumonia, St. aureus, Salmonella enteritis, P. aeruginosa, S. maltophilia* and other opportunistic pathogens of humans and animals. Field tests have shown that the developed TA derivatives are not toxic for fish (Ageyets et al., 2019).

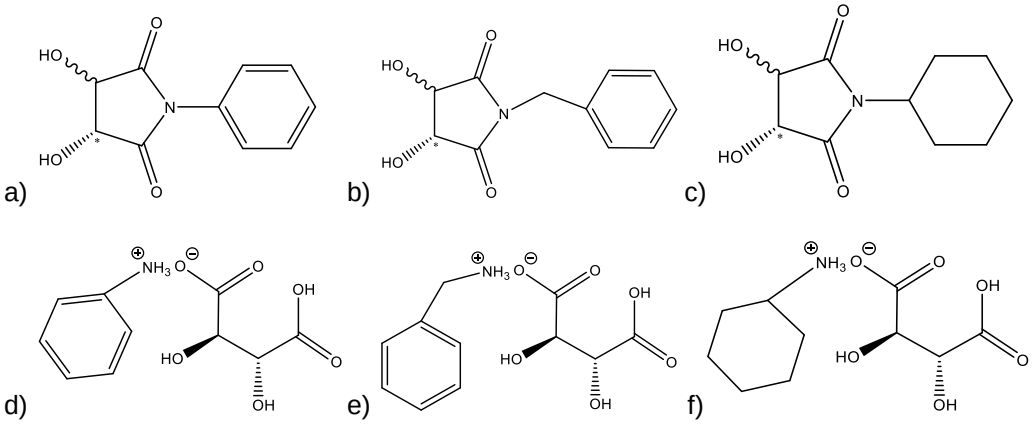


**Figure 1.** Synthetic derivatives of TA; a – phenylimide (PhI); b – benzylimide (BI); c – cyclohexylimide (CI); d – phenylamine complex salt (PhAS); e – benzylamine complex salt (BAS); f – cyclohexylamine complex salt (CAS).

The obtained results are of particular interest, taking into account the potential ability of *P. taetrolens* and other representatives of the *P. chlororaphis* group to utilize complex salts of TA as a carbon source (Baghdasaryan et al., 2019). This suggests the potential environmental safety of using these compounds as antiphytopathogenic agents.

## MATERIALS AND METHODS

### Tartaric acid derivatives obtaining

TA derivatives were generated according to synthetic methodology (Fricke et al., 2010), which was improved in 'Laboratory of Agrarian Pesticide Creation and The Quality Control' at NPUA (Fig. 2) (Dashchyan et al., 2015).

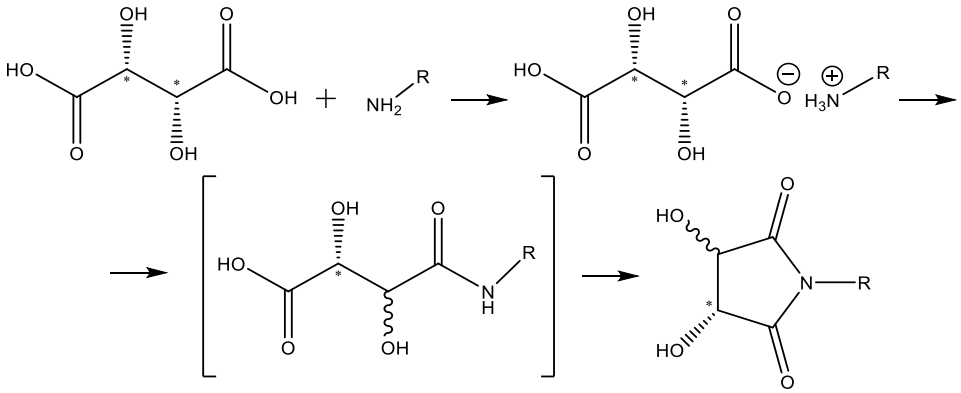

**Figure 2.** TA derivatization principal scheme. R = benzyl-, phenyl-, cyclohexyl-.

**Antibiotic resistance study**

In current research for the microbiological experiments, strains of the National Collection of Microorganisms of Microbial Depository Center (MDC), 'Armbiotechnology' Scientific and Production Center (SPC) of the National Academy of Sciences of the Republic of Armenia (NAS RA) were used. For the research of antibiotic-resistance there were studied 18 strains of *Xanthomonas* following species: *Xanthomonas vesicatoria, X. beticola and Pseudomonas the following strains: Pseudomonas syringae, pv. lachrymans, P. syringae, pv. tabaci*. As the control there were used *E. coli* the following strains: *E. coli DH5α* (non-plasmid sensitive to the used 13 antibiotics), *E. coli DH5α/VOG16 (VOG16* plasmid-containing resistant to kanamycin), *E. coli pUC18 (pIUC18 plasmid containing resistant to ampicillin), E. coli DH5α/PEC7 (PEC7* plasmid-containing resistant to chloramphenicol).

The resistance of the selected strains was defined using solid agarised selective-differential media. Sterilized by autoclaving beef-extract broth and beef-extract agar were used. For qualitative evaluation of antibiotic-resistance of bacteria, the cultivation was carried out by streaking, using 30mm diameter Petri dishes, at 37 °C, under aerobic conditions, on selective-differential media with 50 $\mu g\ mL^{-1}$ content of the following antibiotics: Pcn/Penicillin, Amp/ampicillin, Amx/amoxicillin, Amc/augmentin, Cfx/cefixime, Cro/ceftriaxone (aminopenicillins and cephalosporins of β-lactam antibiotics group), Kan/kanamycin, Stp/streptomycin, Gnc/gentamicin (aminoglycosides), Cip/ciprofloxacin (of fluoroquinolones), Tcn/tetracycline (of tetracyclines), Cam chloramphenicol, also Azm/azithromycin (of azalide macrolides), produced by 'Astoria' (Mikaelyan et al., 2019). The choice of antibiotics was due to their wide applicability in medicine, as well as in agriculture and food industry (Landers et al., 2012).

**TA new derivatives antimicrobial properties monitoring**

Microbiological tests to determine the activity of new TA derivatives were carried out on selective cultivation media. For effect detection on solid cultural media, it was used bilayer media method with some modifications, elaborated in our laboratory (Totten et al., 1982, ThermoFisher, 2008): instead of blood agar it was used meat-peptone agar media. 1.8%-agar containing MPA (meat peptone agar) was poured into Petri dishes (30 mm diameter). Then separately for each tested strain of bacteria there were prepared mixtures of appropriate microbial culture suspension and molten 0.7% agar containing MPA at 37 °C, in sterile test tubes. On surface of solid 1.8%-MPA cultivation media in Petri dishes, the mentioned mixture of suspension of the object microbial strain (~$10^8$ CFU) with 0.7% - agar containing MPA was added as the second layer, for the uniform distribution of microbial culture. After the solidifying of second layer, 3 mcL of each of TA synthetic derivatives (benzylimide, cyclohexylimide, phenylimide, cyclohexyl amine complex salt, benzyl amine complex salt, and phenyl amine complex salt) in concentrations: 0.001M, 0.01M, 0.025M, 0.05M, 0.1M, 0.5M were sterilely added. Solutions were prepared in water for complex amine salts, and in DMSO (dimethyl sulfoxide) for imides, due to their solubility features. The appropriate controls were considered as micro-drops of sterile water and DMSO. The growth of microorganisms under aerobic conditions at temperature optimum of a given species (37 °C) on appropriate cultivation media containing test compounds was visually registered within a period: 12 h, 24 h, 48 h, 76 h. The sterile zones of bacterial growth

inhibition in area of tested compound effect were calculated visually in mm. As a positive control it was considered the growth of the studied bacteria on same cultural media upon the same conditions with sterile pure solvents (water for amine complex salts and dimethyl sulfoxide for imides), without an addition of testing compound. (Eswaranandam et al., 2004).

Determination of minimum inhibitory concentrations were defined according to the generally accepted methods by multiple dilution procedures for each substance beginning from 0.5M to 0.0001M (Andrews, 2001).

**Genetic study of observed *Pseudomonas* and *Xanthomonas* resistance mechanisms**

For the definition of differences in resistance diapason of the studied strains to antibiotics and TA new derivatives, the series of genetic analysis were carried out. Genetic research of the studied bacteria was carried out by isolation of total and plasmid DNA from cells of various strains of phytopathogens, and 0.8–2.5% agar gel-electrophoresis. Plasmid analysis of sensitive and resistant strains of phytopathogens was carried out by electrophoresis and transformation (Thean et al., 2021). As positive control there were used: *E. coli DH5α/VOG16, E. coli pUC18, E. coli DH5α/PEC7; as negative control: E. coli DH5α.*

Mechanism of resistance was studied by the detection of resistance dependence to antibiotic concentration, as well as by Polymerase Chain Reaction (PCR) analysis of genes of antibiotic modifying enzymes (Table 1) (Dumas et al., 2006).

**Table 1.** Conditions of PCR analysis of the studied bacteria resistance

| Gene primer | Subsequence | Temperature regime ($T_{lid}$ 96 °C), |
|---|---|---|
| *blaOXA-10* | F:5'TATCGCGTGTCTTTCGAGTA3'; R:5'TTAGCCACCAATGATGCCC3' | 94 °C-150s, 14 cycles: 94 °C-45s, 63 °C-45s, 72 °C-90s, 22 cycles: 94 °C-45s, 59 °C-45s, 72 °C-90s. |
| *aph(3')VI* | F:5'AGGTGACACTATAGAATACGGAAACA GCGTTTTAGAGC3'; R:5'GTACGACTCACTATAGGGAGGTTTTG CATTGATCGCTTT3' | 94 °C-150s, 14 cycles: 94 °C-45s, 66 °C-45s, 72 °C-90s, 23 cycles: 94 °C-45s, 63 °C-45s, 72 °C-90s. |
| *aac(6')II* | F:5'AGGTGACACTATAGAATATTCATGTC CGCGAGCACCCC3'; R:5'GTACGACTCACTATAGGGAGACTCTT CCGCCATCGCTCT3' | 94 °C - 150s, 14 cycles: 94 °C-45s, 64 °C-45s, 72 °C-90s, 23 cycles: 94 °C-45s, 61 °C-45s, 72 °C-90s. |
| *pCAT639* | F:5'AGGGACGACGGTCATATGGGCAAC3'; R:5'CCTTCGTCCAAGCTTCAGGCCGT3' | 94 °C-150s, 14 cycles: 94 °C-45s, 66 °C-45s, 72 °C-90s, 23cycles: 94 °C-45s, 63 °C-45s, 72 °C-90s. |

The presence of aminoglycoside, amphenicole and β-lactam modification enzymes genes in phytopathogens genome was studied via PCR analysis. The genes of the following enzymes (which are widespread in representatives of the same genera, isolated form clinical samples) were identified: *aph(3')IV, aac(6')II, catB7, blaOXA-10*. The gene *blaOXA-10* is responsible for synthesis of to β-lactamase OXA-10, the gene *catB7* is responsible for chloramphenicol acetyltransferase CATB7. The genes *aph(3')IV* and

*aac(6')II* are responsible for synthesis of enzymes of aminoglycoside antibiotics: modification: aminoglycoside N-acetyltransferase AAC and O-phosphotransferase APH, respectively.

**The resistance transmission study**

The transformation was carried out by the method of obtaining of chemically competent cells, according to Mandel, using low-temperature centrifugation in presence of calcium chloride, with a modification developed in the laboratory of ecological safety of the SPC 'Armbiotechnology', NAS RA (Fig. 3) (Babayan et al., 2020a, 2020b).

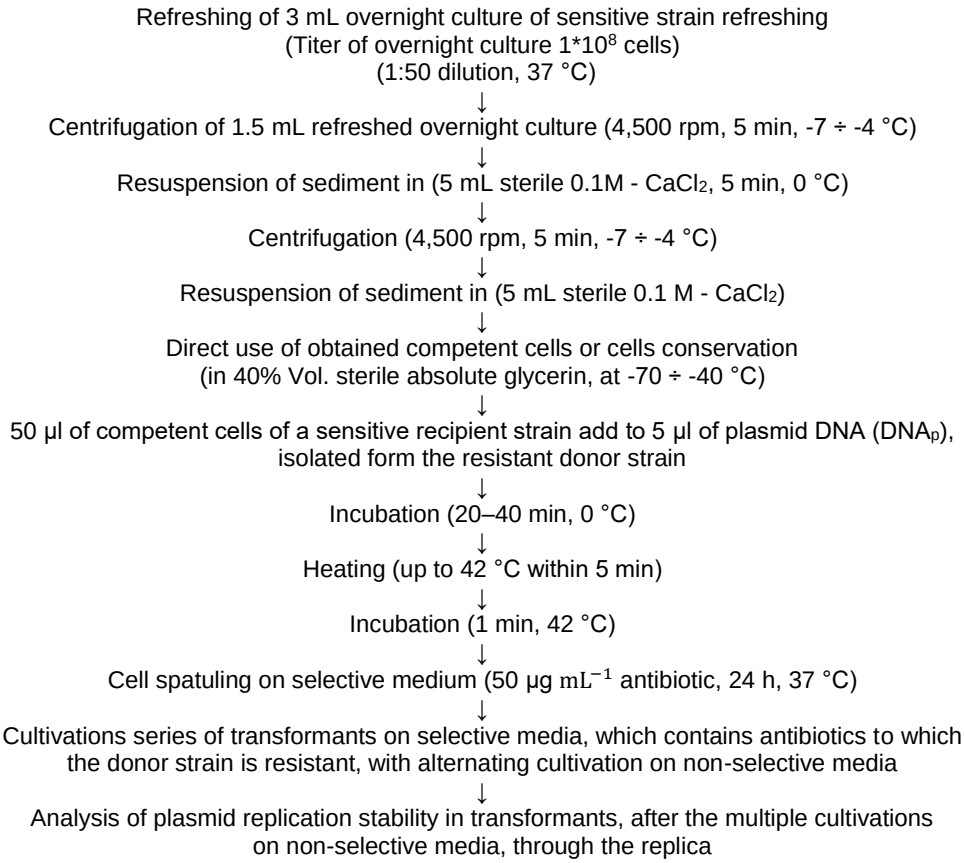


**Figure 3.** Scheme of transformation of strains, sensitive to TA derivatives by plasmids, isolated from strains, resistant to these compounds.

**Statistical assessment**

All the experiments were carries out in 5 series of 3 repeats for each probe. MS Excel was used for data analysis of TA synthetic derivatives antimicrobial effect evaluation. The data of growth inhibition zones are given in mm (Fig. 4–6), SEM (Standard Error of the Mean) were ± 0.23–0.37. Significance was tested by applying Student *t*-test and estimated as *p*-value less than 0.05.

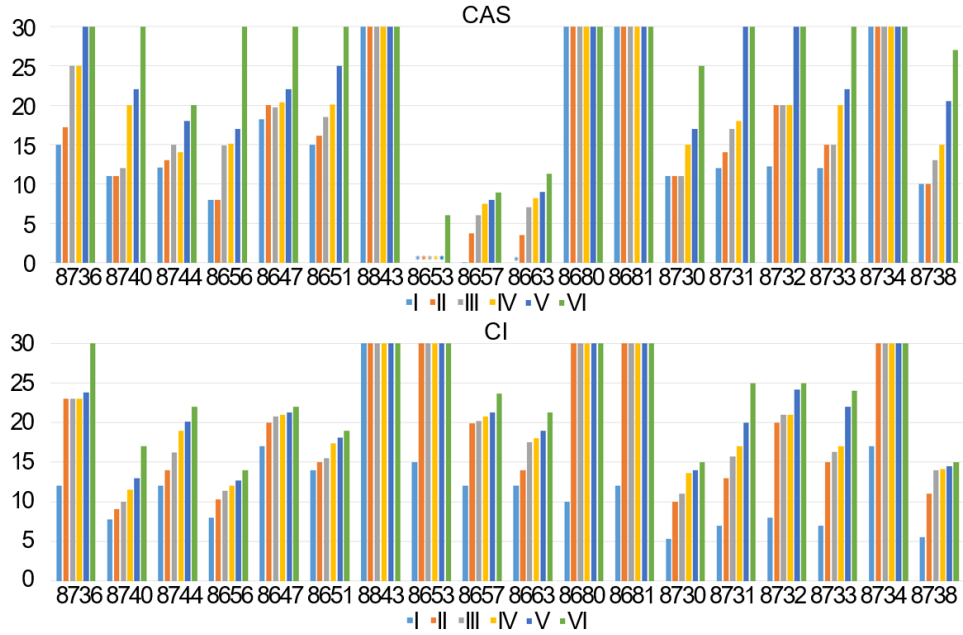


**Figure 4.** Antimicrobial effect of Cyclohexylimide (CI) and Cyclohexyl amine complex salt (CAS) of TA against *Pseudomonas* and *Xanthomonas* different strains. Concentrations: I – 0.001M; II – 0.01M; III – 0.025M; IV – 0.05M; V – 0.1M; VI – 0.5M; '*' – absence of effect. The values of inhibition zones are presented in mm and are average means of 15 independent experiments.

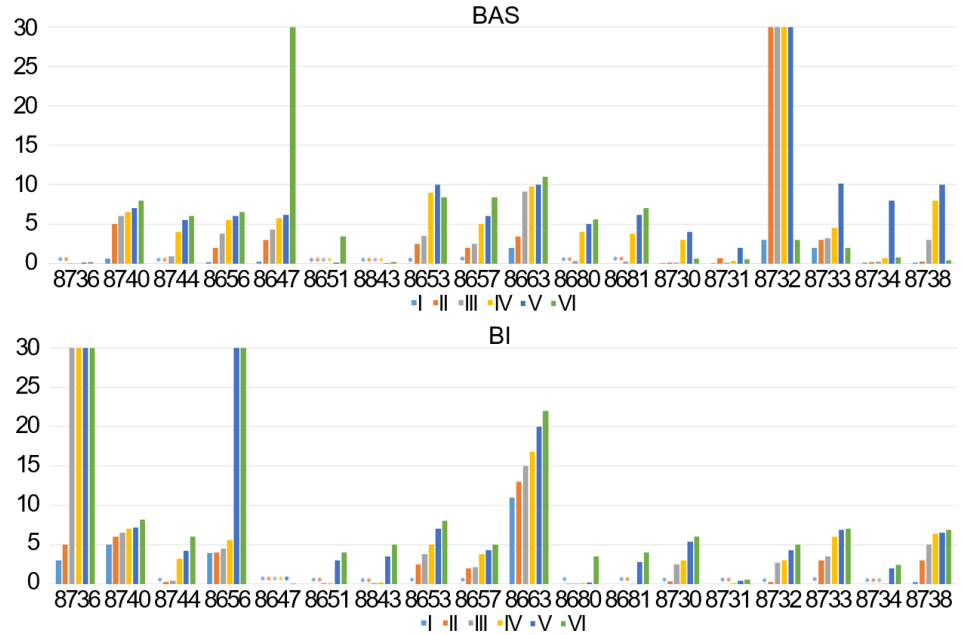


**Figure 5.** Antimicrobial effect of Benzylimide (BI) and Benzyl amine salt (BAS) of TA against *Pseudomonas* and *Xanthomonas* different strains. Concentrations: I – 0.001M; II – 0.01M; III – 0.025M; IV – 0.05M; V – 0.1M; VI – 0.5M; '*' – absence of effect. The values of inhibition zones are presented in mm and are average means of 15 independent experiments.

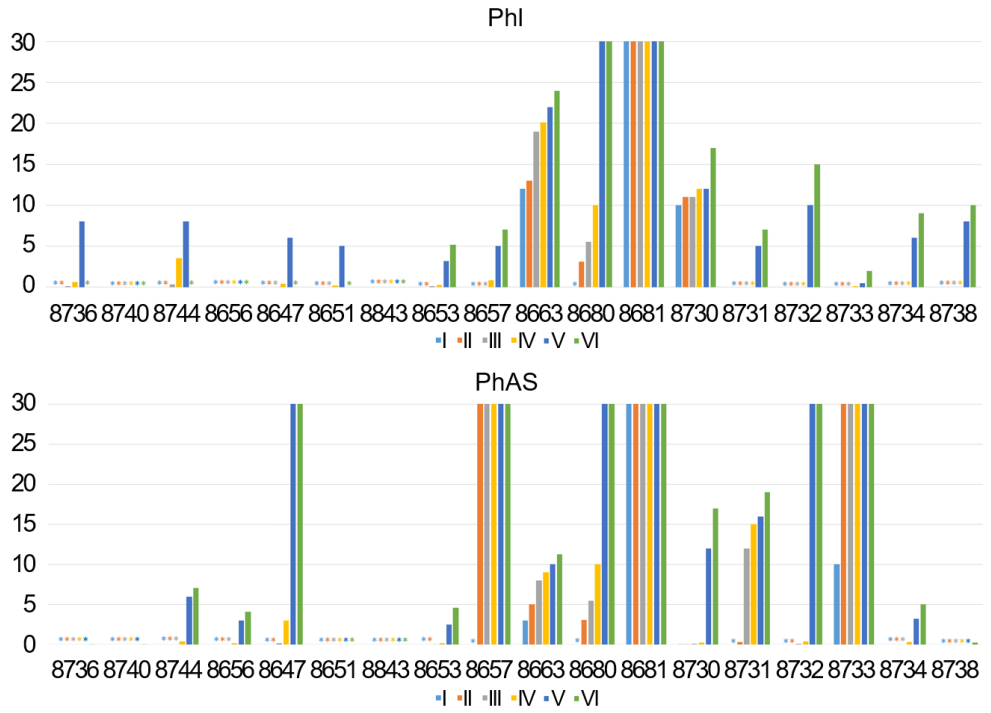


**Figure 6.** Antimicrobial effect of Phenylimide (PhI) and Phenyl amine complex salt (PhAS) of TA against *Pseudomonas* and *Xanthomonas* different strains. Concentrations: I – 0.001M; II – 0.01M; III – 0.025M; IV – 0.05M; V – 0.1M; VI – 0.5M; '*' – absence of effect. The values of inhibition zones are presented in mm and are average means of 15 independent experiments.

## RESULTS AND DISCUSSION

The data obtained during the study of antibiotic resistance of group of phytopathogenic bacteria of various *Pseudomonas* and *Xanthomonas* species are summarized in Table 2. The resistance study of *X. vesicatoria* and *P. syringae* 19 native soil strains to 13 antibiotics of different classes and generations (including antibiotics of the third generation, such as ceftriaxone) revealed a wide diversity of resistance diapason in most strains. The presence of pan-drug resistant and multi-drug resistant representatives was noted.

Table 2 shows that majority of the studied strains are resistant to various antibiotics, including tetracycline and streptomycin, which are widely used in agriculture (Adam et al., 2008). In subsequent experiments, the studied bacterial strains were tested for the effect of various derivatives of TA on solid cultural media, what is presented at figures 4–6 (the detailed method is given in materials and methods).

**Table 2.** Resistance of phytopathogenic *Pseudomonas* and *Xanthomonas* from the National Collection of Microorganisms of MDC, SPC 'Armbiotechnology' NAS RA. Species name (A – *P. syringae, pv. lachrymans*, B – *P. syringae, pv. syringae*, C – *X. vesicatoria, D – P. syringae, pv. tabaci, E – X. beticola*), 50 μg $mL^{-1}$ antibiotic content: 1 – Kan, 2 – Stp, 3 – Gnc, 4 – Cam, 5 – Amc, 6 – Amx, 7 – Amp, 8 – Pcn, 9 – Cfx, 10 – Cro, 11 – Tcn, 12 – Azm, 13 – Cip; control – positive control on a full-fledged cultivation environment; '+' – growth, '-' – inhibition of growth, '- *'- single colonies after the third day of cultivation

| Strain | | Antibiotic resistance | | | | | | | | | | | | | Control |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phytopathogen | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | |
| *8730* | A | - | - | - | - | + | + | + | + | + | + | - | + | + | + |
| *8731* | | - | - | - | + | + | + | + | - | -* | + | - | - | + | + |
| *8732* | | - | - | -* | - | - | + | - | - | + | + | - | - | - | + |
| *8733* | | + | + | - | + | + | + | + | + | + | + | - | - | - | + |
| *8734* | | -* | - | - | - | - | + | - | + | + | + | - | - | - | + |
| *8742* | | + | + | + | - | - | + | - | + | + | + | + | + | + | + |
| *8738* | | + | + | + | + | + | + | - | + | + | + | + | + | + | + |
| *8736* | B | - | - | + | - | + | + | + | + | + | + | + | - | + | + |
| *8740* | | + | + | + | + | - | + | + | + | + | + | + | + | + | + |
| *8744* | | - | - | - | - | + | + | + | + | + | + | + | + | - | + |
| *8647* | | + | - | + | - | + | + | + | + | + | + | + | + | - | + |
| *8843* | | - | + | - | - | - | - | + | + | - | - | + | - | - | + |
| *8651* | C | - | - | - | - | - | + | - | - | -* | -* | + | - | -* | + |
| *8653* | | - | - | - | - | + | -* | - | - | -* | -* | -* | + | -* | + |
| *8657* | D | + | - | + | - | + | + | + | + | + | + | + | - | - | + |
| *8656* | | + | + | -* | + | + | + | - | + | + | + | + | + | + | + |
| *8663* | E | + | + | + | + | - | + | - | + | + | + | + | + | + | + |
| *8680* | | - | - | - | - | -* | -* | - | -* | -* | - | - | - | - | + |
| *8681* | | - | - | - | - | - | -* | - | - | -* | - | - | - | - | + |

Antibiotic resistance tests were carried out in 5 series of 3 repeats of each particular strain of bacteria. Antimicrobial activity was detected by the evaluation of growth inhibition on media. For all the tested compounds, the results of measurements of inhibition zones were considered as average of at least fifteen independent experiments for each bacterial strain. Significance was considered at $p < 0.05$.

For strains *P. syringae, pv. tabaci* 8657, *X. beticola 8680, 8681, P. syringae 8733* effects of phenyl-substituted complex amine salts were higher than in case of phenylimide ($p < 0.01$). In case of benzyl- functional group containing complex amine salts, the effect was higher then in case of imide form for strains *P. syringae pv. lachrymans 8736, P. syringae, pv. tabaci 8656* ($p < 0.01$), while for strains *P. syringae pv. lachrymans 8736, 8740, 8744*, *P. syringae, pv. tabaci 8656* the imide was more effective growth inhibitor, then the appropriate complex amine salt. For both cyclohexyl- functional group substituted derivatives there were noted the comparable high effects of bacterial growth inhibition for the majority of *Pseudomonas* and *Xanthomonas* studied strains of different species ($p < 0.01$), while for the strains *X. beticola 8653, 8657, 8663 the effect of imide was higher than in case of complex amine salt* ($p < 0.05$). *Due to the results, the antimicrobial effect of TA synthetic derivatives is a function from both substituent chemical nature and individual genetical features of each particular strain. That is why the genetical analyses of antimicrobial resistance mechanisms were carried out.*

According to the observations data, the effective concentrations of cyclohexyl derivatives are lower than the concentrations of benzyl- and phenyl- derivatives both for imides and amine complex salts. According to the results, the minimum inhibitory concentrations (MIC) of tested compounds are differing depends to strain of bacteria (Table 3).

Probably, this is due to the differences in bioavailability of salt and imide forms of these compounds and due to the species-specific features of the permeability systems of bacterial cells. This also might be explained by the affinity of cyclohexyl- (CAS and CI) derivatives to certain species-specific membrane proteins of *Pseudomonas* representatives, what was observed via docking analyses of analogues compounds, had been shown in earlier research (Chang et al., 2015).

**Table 3.** Minimum Inhibitory Concentrations (MIC) of TA new derivatives estimated for *P. syringae, pv. tabaci 8736* and *X. beticola 8681*

| TA derivative | Minimum Inhibitory Concentrations (MIC) | |
|---|---|---|
| | *P. syringae, pv. syringae 8736* | *X. beticola 8681* |
| BI | (0.001M) 6.6 mcg $mL^{-1}$ | (0.0001M) 66 ng $mL^{-1}$ |
| BAS | (0.5M) 3.84 mg $mL^{-1}$ | (0.0001M) 76 ng $mL^{-1}$ |
| CAS | (0.0001M) 74.7 ng $mL^{-1}$ | (0.001M) 7.47 mcg $mL^{-1}$ |
| CI | (0.0001M) 31.8 ng $mL^{-1}$ | (0.0001M) 31.8 ng $mL^{-1}$ |
| PHI | (0.022M) 50 mcg $mL^{-1}$ | (0.001M) 6.18 mcg $mL^{-1}$ |
| PhAS | (0.01M) 618 mcg $mL^{-1}$ | (0.001M) 7.29 mcg $mL^{-1}$ |

During the genetic analyses the presence of plasmids and genes of antibiotic modification enzymes was studied, as well as their localization and grade of transmission ability by the interspecies gene horizontal transfer under selective and non-selective conditions. Studies have shown that some of strains contain plasmids that differ in molecular weight and are capable to transmit antibiotic resistance. The other part of strains has plasmids that are not responsible for transferring of aminoglycosides and β-lactam resistance genes. There are also strains in which no plasmids have been found. For all the studied strains, plasmids with an ability of transmission of resistance to any TA synthetic derivatives were not detected (Fig. 7).


**Figure 7.** Electrophoretic study of DNA of various phytopathogenic *Pseudomonas* and *Xanthomonas* from the National Collection of Microorganisms of MDC, SPC 'Armbiotechnology' NAS RA on 0.8% – 2.5% of agarose gel. 1 – *P. syringae path. lachrymans 8732*; 2 – *X. vesicatoria 8647*; 3 – *P. syringae, path. syringae 8736*; 4 – *X. beticola 8680*; 5 – *P. syringae, path. tabaci 8663*; 6 – *P. syringae, path. tabaci 8665*; 7 – *S. maltophilia 9286*; 8 – *X. beticola 8681*.

The results of the analysis comparing the different genetic properties of *Pseudomonas* and *Xanthomonas* with their resistance to antibiotics of various classes and generations are listed in Table 4. The

presence of plasmids, their ability of antibiotic resistance transmission and its stability in the case of various recipients were studied using appropriate selective media.

Microbiological experiments have shown that in studied group of *Pseudomonas* and *Xanthomonas*, the resistant representatives prevail, most of which are multi-drug resistant or pan-drug resistant. Antibiotic sensitive strains are in the minority, or show lagging growth on an appropriate selective media. It should be noted the high stability of resistance in this group of strains, which were deposited and cultivated on nutrient media without contact with any antimicrobial compound. That resistance maintains as a result of both nucleoid and plasmid genes. Possibly this is caused by the presence of genes of primary metabolism enzymes on their plasmids, what determines the stability of the plasmids under non-selective conditions of cultivation. In that aspect, the strains *X. vesicatoria 8656* and *P. syringae 8740*, which demonstrate the resistance to almost all the used antibiotics, are of particular interest.

**Table 4.** Genetic properties of plasmids of various phytopathogenic *Pseudomonas* and *Xanthomonas*. I – *X. beticola*, II – *P. syringae, pathovar tabaci*, III – *P. syringae, pv. lachrymans*, IV – P. *syringae, pv. syringae*, V – *X. vesicatoria*, '+' – the presence of a feature, '-' – the absence of a feature

| Strain of Bacteria | | Plasmid content | Ability to transfer the genes of resistance to 13 studied antibiotics | Ability to transfer the genes of resistance to TA derivatives |
|---|---|---|---|---|
| *I* | *8680* | - | - | - |
| | *8681* | - | - | - |
| | *8663* | + | + | - |
| *II* | *8656* | + | + | - |
| | *8657* | + | + | - |
| *III* | *8730* | + | + | - |
| | *8731* | + | + | - |
| | *8732* | - | - | - |
| | *8733* | + | + | - |
| | *8734* | - | - | - |
| | *8738* | + | + | - |
| | *8742* | + | + | - |
| *IV* | *8740* | + | + | - |
| | *8744* | + | + | - |
| | *8736* | + | + | - |
| *V* | *8843* | + | - | - |
| | *8651* | + | - | - |
| | *8653* | + | - | - |
| | *8647* | + | - | - |

The results of PCR analysis had shown the *blaOXA-10* gene was found in *P. syringae 8740*. According to the negative results of transformation of sensitive strains *P. aeruginosa 9056, E. coli DH5α* (not containing plasmids), *X. vesicatoria 8647* (according to this study, the plasmids of this phytopathogenic microorganism do not transfer resistance to the given 13 antibiotics) by plasmids of the given strain, the *blaOXA-10* gene is located on the bacterial chromosome of the particular microorganism. Plasmids were also found in *P. syringae 8736, 8656, 8740* and in *X. vesicatoria 8647* by gel electrophoresis and transformation. Some of them are not related to resistance (*P. syringae 8740*) to the studied antibiotics, while the rest of them carry genes of resistance to β-lactam antibiotics. According to the transformation experiments, resistance to aminoglycoside and amphenicole antibiotics in all studied strains of *P. syringae* and *X. vesicatoria* is represented by nucleoid genes (Niu et al., 2015). All the studied strains, which contains the appropriate plasmids are able to transmit antibiotic resistance and are demonstrating stable replication of the particular inserted plasmids, in case of *Pseudomonas* recipients, as well as *Stenotrophomonas*

*maltophilia* (belonging to the same *Xanthomonadaceae* family) recipient. However, in case of *Xanthomonas* and *E. coli* recipients, stability was not observed.

In subsequent experiments it was observed that the effect of TA derivatives does not depend to content of plasmids. Both investigated phenyl- derivatives of TA (PhAS and PhI) don't have an effect on strains containing the *blaOXA-10* gene, while benzyl- and cyclohexyl- derivatives (BAS, CAS, BI, CI) are active against all studied bacteria. However, in contrast to the Tcn-resistant *P. chlororaphis* strains, the growth of similar *P. syringae* and *X. beticola* representatives is suppressed by benzyl- and cyclohexyl- complex salts and TA imides.

Perhaps this is related to differences in the substrate specificity of polyphenol oxidases and other enzymes of Tcn-resistance, which are encoded by the nucleoid, as the characteristics of a particular bacterial species (Sultan et al., 2018). Our studies have shown that the most of *P. syringae* representatives have plasmids, which are responsible for β-lactams resistance transmission. No plasmids were found in *Xanthomonas* representatives. Genes *aph(3')IV, blaOXA-10, aac(6')II,* and *catB7* were also not found. The resistance of these strains is caused by other plasmid and nucleoid encoded genes. Literature data indicate a wide variety of similar genes and their localization in *Xanthomonas* genome (Tamir-Ariel et al., 2007). During the cultivation of transformants constructed on the basis of sensitive strains and plasmids isolated from resistant strains, the stable replication of plasmids on non-selective media was revealed for *X. beticola*, *P. syringe* plasmids in *P. aeruginosa 9056* recipients. In contrast to it, the replication of plasmids in *E. coli DH5α* is stabile only on selective media containing the corresponding antibiotic, the modification gene of which is located on the plasmid.

## CONCLUSIONS

Antibacterial effect of TA new derivatives was studied on 19 model strains of phytopathogens from *Xanthomonas beticola (6 strains), X. vesicatoria and Pseudomonas syringae (13 strains)*, isolated form soil and different in their sensitivity to antibiotics. New synthetic derivatives of TA have demonstrated effect against the range of phytopathogenic bacteria of *Pseudomonas* and *Xanthomonas* genera. Cyclohexyl- and benzyl- imides and complex salts of TA are more effective than phenyl-derivatives. The effect of the discussed TA derivatives on resistant strains *P. syringae*, *X. beticola* and *X. vesicatoria* species does not correlate with the presence of plasmids and genes of β-lactam, amphenicol and aminoglycoside modification enzymes in genome. Thus, the mechanisms of effect of the discussed compounds are related with another molecular targets in cells. And the impossibility of transfer of resistance to TA all the discussed derivatives by plasmids was demonstrated. It makes these substances recommended for other further research for the elaboration of new classes of potentially ecologically safe anti-phytopathogenic drugs for crop and garden trees protection. For that aim, more detailed *in silico, in vitro, in vivo* eco-toxicological research are under evaluation. And further more detailed research of the discussed substances effects on bacteria, as well as antimicrobial resistance mechanisms features influence studies needed as a next step of research.

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