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Molecular Identification of Invasion Diseases Agents of *Acer negundo* L. in South Kazakhstan

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The *Acer negundo* L. is one of native species of flora in Kazakhstan and forms a basis of dendroflora in all settlements. For last 10 years development of unknown diseases of *Acer negundo* L. has been registered. By the molecular-genetic analysis it is established, that agents of these diseases are microfungi of *Fusarium solani* and *Fusarium oxysporum.* Identity number of nucleotide sequences of their ITS regions with controller FJ914886.1 and EU625403.1 from the international bank (GeneBank) is 100, 0 and 99, 0 %, respectively. Currently, *Bacillus thuringiensis 4ant* bacteria have been isolated, which are capable of inhibiting the growth of the phytopathogenic fungus, the causative agents of trunk disease infecting *Acer negundo* L. The *Bacillus thuringiensis 4ant* strain's antagonistic properties bespeaks its practical value as a useful agent in the biological struggle against diseases that cripple the dendroflora of the South of Kazakhstan.

Key words: *Acer negundo* L., verticillium wilt, anthracnose, phytopathogen, *Fusarium*, micromycetes, antagonists, molecular-genetic analysis, *Bacillus thuringiensis*.

There are about 128 species, most of which are native to Asia[1], with a number also appearing in Europe, North Africa and North America. Only one species, the poorly studied *Acer laurinum*, is native to the Southern Hemisphere[2]. 54 species of maples meet the International Union for Conservation Nature criteria for being endangered in their native habitat. *Acer negundo* is one of the native flora in Kazakhstan and forms the basis dendroflora in all localities. Maple is highly resistant to dry climate conditions, which explains its ubiquity. They are susceptible to a number of diseases, infections and other conditions. Some fungal infections damage the branches, limbs and roots, but others may eventually kill the tree. Various fungal diseases can infect maples and these diseases, including anthracnose, leaf spot, powdery mildew, leaf blight, verticillium wilt and rot. Anthracnose causes black spots with a tarlike appearance on infected leaves and occurs in wet, humid conditions[3-5]. Leaf spots are identified by the discolored, irregularly shaped spots that develop on the foliage. Powdery mildew is one of the few fungal diseases that doesn't require moisture to germinate and spread. It causes a powder like growth to form on buds, stems and leaves, and infected foliage will curl and distort. Rots cause leaves to wilt and fall from the branches,

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stunts new growth, then branch dieback occurs and the tree begins to decline[6]. Nevertheless, epiphytotic development of especially dangerous diseases of species has not been recorded in Kazakhstan for last years. Unknown diseases of maple trees in Kazakhstan dendroflora became evident last 10 years. It was established, that this process was connected with invasion of new for the country species of pests-xilophages as *Monochamus urussovi* F., *Monochamussutor* L., *Acanthocinusaedelis* L., *Cetoniaaureta* L., *Cossuscossus, Xanthogalerucaluteol, Archipsxylostena* which larvae are the basic distributors of phytopathogenic microflora spores[7, 8].

Acer negundo fungus is present in diseased maple trees and should be prevented from occurring[9-14]. Care of maple trees should be undertaken to avoid this disease that these trees could experience. This disease is normally caused by insects and pests and usually requires immediate treatment to overcome the problem. This fungus is indicated by purple brown spots on the maple tree leaves and it is thought that the trees are more prone to suffer with this in cold and wet weather. As this fungus can damage the buds and twigs of the tree if left untreated, it is essential to remove any affected foliage of the maple tree as soon as this is noticed[15].

This condition usually occurs when there are long periods of cold and wet weather. Suffered areas may include small dark spots and irregular shape of the dead and brown areas on the leaves. Leaves usually fall in early spring, followed by a second set of leaves, which will also die. Affiliates can develop rust that can encircle branches and kill them[16]. The disease is perpetuated because the fungal spores over-winter in dead leaves. When there is a prolonged wet spring, the spores have a perfect breeding ground. The spores are carried by the wind to other trees. Once infected, the disease can over-winter in the host plant in the infected branches and twigs. Invasive disease causes processes of decay and cancer tumours of trunks maple, that finally, leads to its blight. In the researches conducted earlier the specific structure of phytopathogens, causing trees disease, was not determined. Our studies deal with the quest for effective strains of antagonistic microorganisms against the causative agent of *Acer negundo* L. trunk disease in the arid climate of the South of Kazakhstan and molecular-genetic identification of invasion diseases agents of maple trees was a task of our researches. In conditions of the ecologization of the process of protecting plants, priority is given to biological methods of struggle, which is based on using the microbial antagonism phenomenon[17]. The use of biological preparations in the struggle against the causative agents of diseases of dendroflora is a promising alternative to chemical means of fighting them in the urban ecosystem[18]. At present, despite the availability of numerous bio-preparations employed already, the quest for new strains of antagonists adapted to specific soil/climactic conditions remains a topical issue to investigate[19-24]. Therefore, isolating and identifying anti-fungus strains of antagonistic microorganisms is drawing increasingly more interest.

MATERIALS AND METHODS

As research material there were 2 strains of micromycetes (2C and 5.2y), isolated of the effected tissue of trunk and leaf *Acer negundo* (figure 1) and *4ant* strain of bacteria of the *Bacillus* genus, which was isolated from soil samples picked in seven districts of the South Kazakhstan Province. The pure culture assumed phytopathogens was received by a method of exhausting seeding on selective nutrient media with observance of aseptic working conditions. Isolation and account of micromycetes were carried out by conventional mycological methods[25].

Identification of micromycetes was carried out by a method of direct determination of nucleotide sequences of their ITS regions *(of intergene* transcribed *region*) and with the subsequent comparison of their identity with nucleotide sequences deposited in the international database (GeneBank) of samples, and also construction of phylogenetic trees with nucleotide sequences of referential strains. The *4ant* strain of antagonistic bacteria was identified using the genic analysis of the 16S ribosomal RNA (16S rRNA) gene sequence. The genomic DNA was extracted from the *4ant* strain of *Bacillus thuringiensis* as the matrix for the polymerase chain reaction, and the 16S rRNA gene was amplified through the PCR. To determine the 16S rRNA sequence, the genomic

DNA was grown using the CTAB/NaCl method (Ausubel *et al*., 1989)[26]. DNA isolation was conducted in a buffer solution @" 8,0; 100 mMTris-HCl, 1,4 M NaCl, 20 mM EDTA, 2 % CTAB and K 100 mg/ml proteinase. The culture, after centrifuging and removing of supernatant, triturate to a powder condition with addition of liquid nitrogen. Then in 100 µl of suspension incubate the culture for 18 hours, adding 500 µl of corresponding buffer. Clear suspension adding 750 µl of chloroform/isoamyl alcohol (24/1), stirring and with subsequent centrifuging at 12 000 rpm for 10 minutes. Clear a water phase of culture repeatedly with phenol/chloroform isoamyl alcohol (24/24/1). DNA precipitating was conducted with 0, 6 volumes of isopropyl alcohol and then centrifuge culture repeatedly. Wash DNA sediment with 70% ethyl alcohol, with subsequent centrifuging and removing of a liquid phase. Dry the received sediment in the open air for 15 minutes. Samples of DNA dissolve in 100 µl of single buffer " and store at 20°C. DNA concentration measure with spectrophotometer Nano Drop at a wavelenth of 260 nm[27].

A fragment of the 16S rRNA gene sequence of the strain under study, which had been amplified by PCR, was compared with standard 16S rRNA gene sequences of other bacteria provided by the BLAST NCBI database.

The study revealed that partial sequences of the 16S rRNA genes of the strain under study and *B.thuringiensis* bacteria were completely identical. Thus, the object of our study was identified as a new strain of *B.thuringiensis* bacteria. The next step dealt with a more in-depth investigation of its antagonistic capacity against various kinds of phytopathogenic fungi.

The antagonistic capacity of the 4ant strain. It was established that the *4ant* strain possesses high inhibiting activity against a number of phytopathogens – *F.solani, Fusarium oxysporum* (Table 1). Figure 2 illustrates the strain's effective fungicide effect against the *F.solani* and *F.oxysporum* fungus in a model experiment with a pure culture of a phytopathogen.

RESULTS AND DISCUSSION

Studying of micro- and macro morphological features of micromycetes in pure cultures showed, that 2 studied by us strains belong to the class imperfect. These strains, with well-developed myceliums, in a nutrient media formed convex and velvet colonies of pink color, in diameter 1,3 -1.8 sm. Micro - and macro conidiums of this fungus are distinctly bent, in size 15-60 µm having various number of partitions with expressed basal cell. On 20th day of experience in fungus colonies of chlamydospore development was established, in size approximately 18 µm in diameter which had thick and light brown color of a cover. On the basis of a complex of morphological features these strains fungi were preliminary identified[28], as the representatives of *Fusarium* (Fig. 3).

Final identification of investigated 2 fungi strains was conducted on the basis of results of the molecular-genetic analysis (table 2).

The research results from references confirm that the majority of *Fusarium* are agents of diseases of the most various species of plants. Thus it is revealed, that fusariums of the same species can damage plants of the various families, causing the various pathological phenomena - decay of roots, seeds, fruits, and also the general

Fig. 1. a - Diagnostic powdery growth on upper surface of a *Acer negundo* leaf, b - Appearance of the diseased of *Acer negundo L.*

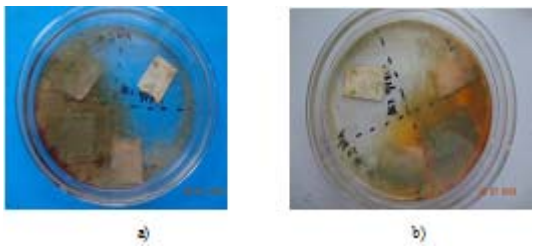

Fig. 2. The antagonistic effect of the *B.thuringiensis* strain, *4ant*, on the growth of *Fusarium solani (a) and of Fusarium oxysporum.*

Table 1. The antimicrobial effect of the supernatant, containing no cells, of *B.thuringiensis 4ant* against indicator bacteria.

Indicator strains	Incubation temperature	Antagonistic activity
Fusarium solani	27 °C	++
Fusarium oxysporum	25 °C	+

Zones of inhibition: +, e 4mm; ++, e 8mm

1) 2y strain colony species of *Fusarium* fungus, isolated of the effected tissue of *Acer negundo*;
2) 5.2y strain colony species of *Fusarium* fungus in pure culture.

Table 2. Results of molecular-genetic identification of micro fungi strains from the effected tissue of *Acer negundo* trunk using the analysis method of nucleotide sequences of ITS region

The name of the strains	Identification of nucleotide sequences in the international database (http://www.ncbi.nlm.nih.gov/) with algorithm BLAST		
	Accesion number of GeneBank	The strain name	% identification
1sample 2y	FJ914886.1	*Fusarium oxysporum*	99
2 sample 5.2y	FJ914886.1	*Fusarium solani*	100

suppression and premature wilt. In most cases they cause diseases of root systems of plants in the form of decay and tracheomycosis vascular wilt of plants. *F. avenaceum, F. solani, F. culniorum, F. gibbosum, F. semitectums cause* root rotting of herbs as potato, peas, kidney bean, melon, water-melon and tomato. And species as *F. graminearum* and *F. nivale* are agents of diseases of grains as vascular wilt and a snow mold [29-31]. Possibility of damage of woody plants by these fungi is proved with results of many researches. For example, a general decline of the tree is also often observed Incidence of bleeding cankers on maples may increase when trees experience stress as a result of heavy salting[32]. These reports are the result of consistent isolation of these pathogens from the cankers and not from inoculation experiments. Some trees are able to successfully compartmentalize the infection if it is limited[33]. Caroselli (1953) also notes that removal of infected tissue has not been a successful control strategy. Fungicides may be effective but are not often economically feasible. Bleeding cankers caused by *Fusarium solani* were reported on sugar and red maple by Wood and Skelly. Initial black exudates are followed by the development of sunken areas and eventually the bark breaks away. Cankers developed when isolates of the pathogen were inoculated into healthy maples[25]. Inoculations conducted during the spring produced black exudates by the next fall. Weidensaul found that *F. solani* was easily cultured from the bark of healthy

sugar maples and hypothesized that any wound could be the site of introduction of the pathogen to the inner bark. If wounding occurs during the dormant season, then a canker develops. Once the tree resumes growth in the spring, it is able to contain the pathogen and eventually close over the canker. Drillias *et al.* (1982) describe a basal canker of sugar maple caused by an unidentified *Fusarium* species[34]. Seemingly healthy bark at the base of the trunk exudes sap and covers necrotic, dark reddish brown tissue that extends into the wood. A *Fusarium spp.* was consistently isolated from bark and wood chips taken from the margins of these cankers. Phloem and cambium underlying the symptomatic area are discolored in an elongated, tapering shape. Nevertheless, the disease is generally considered to be spread by the narrow winged tree cricket[35].

Thus, results of our and known from references researches prove, that isolated by us fungi strains possess phytopathogenic properties. In conditions of arid climate in Southern Kazakhstan these pathogens damage tissue subcortical bast of *Acer negundo L.* trunks.

As was pointed out above, the identified strain of bacteria *B.thuringiensis 4ant* demonstrated high fungicidal capacity in model experiments, which bespeaks its practical value. This assertion jibes with research study findings available in scientific literature, in which it is shown that certain strains of bacteria and *B.thuringiensis* can synthesize over 60 different types of antimicrobial secondary metabolites, including polypeptides, which are effective fungicides[36].

CONCLUSION

Thus, the results of the molecular-genetic analysis established, that agents of invasion diseases of *Acer negundo* L. trunks in South Kazakhstan are microfungi of Fusarium - *F.solani* and *F.oxysporum* which are characterised as widespread phytopathogens on many species of herbaceous 8 woody plants. Identity of nucleotide sequences with controller numbers FJ914886.1 and EU625403.1 in international bank GeneBank is 100,0 and 99,0 %, respectively. The strain of bacteria isolated from soil samples and identified as *B.thuringiensis 4ant* is an effective antagonist for the causative agent of *Acer negundo* L. trunk disease, *F.solani* and *F.oxysporum*.

REFERENCES

1. Dirr, M.A. and C.W. Heuser, The reference manual of woody plant propagation: from seed to tissue culture.Varsity Press, 1987; **5** .
2. Kukor, J., D. Cowan and J. Martin, The role of ingested fungal enzymes in cellulose digestion in the larvae of cerambycid beetles. *Physiol. Zool*, 1988; **61**.
3. Eyre, F.H., Forest cover types of the United States and Canada. Society of American Foresters, Washington, 1980; **148**.
4. Gibbons, A., Stalking the wild asparagus, David McKay Co., New York, 1962; 336p.
5. Greco, A.M., J.E. Winstead and F.R. Toman, Chlorophyll levels as ecotypic characters in boxelder seedlings. *In Forty-first Transactions of Kentucky Academy of Science*, 1980; **81**.
6. Green, G.H., Trees of North America. Edwards Bros., *Ann Arbor*, 1934; **11**. 344 p.
7. Hall, B.A., Variability in the floral anatomy of Acer negundo. *American Journal of Botany*, 1954; **41**.
8. Harlow, A., M. William, C. Ellwood, A. Harrar and M. Fred, Textbook of dendrology. McGraw-Hill, 1979; **4**. 510 p.
9. Hepting, M. and H. George, Diseases of forest and shade trees of the United States. U.S. Department of Agriculture, 386. *Agriculture Handbook*, 1971; **25**. 685 p.
10. Hosner, J., Relative tolerance to complete inundation of fourteen bottomland tree species. *Forest Science*, 1960; **6**.
11. Forest Science Morewood, W.D.; Neiner, P.; Hoover, K. Evaluating Asian Longhorned Beetle.
12. Adult Preference and Larval Performance Among Commonly Planted Landscape Trees.
13. W.D.; Neiner, P.R.; Sellmer, J.C.; Hoover, K. Behavior of adult *Anoplophora glabripennis* on different tree species under greenhouse conditions. Processeding of 13th Metropolitan Tree Improvement Alliance and Landscape Plant Development Morewood.
14. Stevens, P. F. (2001 onwards). Angiosperm Phylogeny Website. Version 9, June 2008 [and more or less continuously updated since].
15. Jones, R.K. and D.M. Benson. *Diseases of woody ornamentals and trees in nurseries*. The American Phytopathological Society, 2001; St. Paul, MN.482p.
16. Baird & Taylor's reference to this species is

regarded as a cultivated tree or as an error: Manual Public Schools of Clark County, Ind. 1878-9, page 62.
17. Izhevsky, S.S., The Significance of the Zonality Factor in Applying the Biological Method of Plant Protection in the Territory of the Russian Federation. Trudy VIII Mezhdunarodnogo kongressa po zashchite rastenii, 1975; **8**: 39-45.
18. Deacon, J.W., Significance of ecology in the development of biocontrol agents against soil-borne plant pathogens. *Biocontrol Science and Technology*, 1991; : 5-20.
19. Yakimenko, .. and I.D. Grodnitskaya, The Impact of Fungi of the Trichoderma Genus on Soil Micromycetes Causing Infectious Root Rot in Conifers in the Forest Nurseries of Siberia. *Microbiology*, 2000; **6**: 50-54.
20. Raaijmakers, J.M., ". Vlami, and J.". de Souza, Antibiotic production by bacterial biocontrol agents. *Antonie van Leeuwenhoek*, 2002; **81**: 537-547.
21. Wilson, C.L. and M.E. Wisniewski, Biological control of post-harvest diseases of fruits and vegetables: alternatives to synthetic fungicides. *Annual Review Phytopathology*, 1991; **10**: 172-177.
22. El-Ghaouth, A., Biologically-based alternatives to synthetic fungicides for the control of post harvest diseases. *Journal of Industrial Microbiology and Biotechnology*, 1997; **17**: 160-162.
23. EL-Banna, N.", Effect of carbon source on the antimicrobial activity of Corynebactirumkutscheri and Corynebactriumxecroris. *Afr J Biotechnol*, 2006; **5**: 833-835.
24. Stein, T., *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Molecular Microbiology*, 1998; **4**: 845-857.
25. Vines, Robert A. Trees, shrubs, and woody vines of the Southwest. University of Texas Press, Austin, 1960; TX. 1104p.
26. Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A.Smith and K. Struhl. Current Protocols in Molecular Biology. New York: John Wiley and Sons, pp. 37-44
27. Williams, H. D., Jr., and J. E. Winstead. Population variations in seed germination and stratification of *Acer negundo* L. p. 43-48. In Thirty-third Transactions of the Kentucky Academy of Science 1972.
28. Williams, R. D., Jr., and J. W. Winstead. Populational variation in weights and analysis of caloric content in fruit of *Acer negundo* L. Castanea, 1972; **37**:125-130.
29. Winstead, J. E. Tracheid length as an ecotypic character in *Acer negundo* L. *American Journal of Botany*, 1978; **65**:811-812.
30. Zolotov, H. N. [Revegetation of eroded gullies by natural seeding from maple (in adjacent plantations). Lesnoe Khoziaistvo 11:68-79. [Original not seen. *Abstract in Forestry Abstracts*, 1958; **20**(3146).
31. Pinkerton J.N., Johnson K.B., Mehlenbacher S.A., Pscheidt J.W., Susceptibility of European hazelnut clones to EFB. *Plant Disease*, 1993; **77**: 261-266.
32. Postman J.D., Johson K.B., Maas J.L., Davis R.E., The Oregon hazelnut stunt syndrome and phytoplasmas associations. *Acta Horticulturae*, 2001; **556**: 407-410.
33. Rovira M., Aramburu J., Incidence of apple mosaic ilarvirus (ApMV) in Catalogna (Spain) and its effect on "Negret" hazelnut. *Acta Horticulturae*, 2001; **556**: 509-512.
34. Santori A., Belisario A., La necrosi grigia della nocciola: approfondimenti su eziologia ed epidemiologia. *Informatore Fitopatologico, supplemento Terra e Vita*, 2008; **49**: 18-23.
35. Decock C., Figueroa S.H., Robledo G., Castillo G., *Fomitiporia punctata* (Basidiomycota, Hymenochaetales) and its presumed taxonomic synonyms in America: taxonomy and phylogeny of some species from tropical/subtropical areas. *Mycologia*, 2007; **99**: 733-752.
36. Dittberner, Phillip L.; Olson, Michael R. The plant information network (PIN) data base: Colorado, Montana, North Dakota, Utah, and Wyoming. Washington, DC: U.S. Department of the Interior Fish and Wildlife Service. 1983; p.786