RESEARCH PAPER

Microscopic fungi antagonistic to chestnut blight– *Cryphonectria parasitica* (Murrill) Barr.

Irina Danelia[1], Nino Zakariashvili[1], Gulnara Badridze[*1], Lali Kutateladze[2],

Maia Jobava[2], Nino Lomidze[1], Ketevan Benashvili[1]

[1] *The Technical University of Georgia, Faculty of Agrarian Sciences and Bioengineering, Guramishvili 17, Tbilisi, Georgia*

[2] *Agrarian University of Georgia, Institute of Biochemistry and Biotechnology, Agmashenebeli alley, Tbilisi, Georgia*

Publication date: May 30, 2018

Abstract

The extent of cryphonecrosis among the chestnut populations of three Imeretian (west Georgia) villages: Darka, Eto, and Chala has been evaluated. 23 strains of *Cryphonectria parasitica* (Murrill) Barr (syn. *Endothia parasitica* (Murrill) were isolated and identified from the bark of sick trees. The collection of strains of the plant pathogen fungus has been created. The strategy of the struggle against the chestnut blight, based on the application of antagonistic to *C. parasitica* microscopic fungi, has been elaborated. For this purpose 50 strains of different microscopic fungi were isolated and identified (till genus) from the soil samples picked just under the stems of sick trees of above mentioned locations. The dominating genera of micromycetes in forest brown soils have been revealed. Strong biological antagonists of the plant pathogenic fungus, belonging to genera *Penicillium*, *Trichoderma* and *Aspergillus* have been selected on the base of the investigation of antagonistic activity of the "aboriginal" flora of studied soils. The collection of antagonistic to *C. parasitica* microscopic fungi, among them of new biological agents, has been created. The vegetative compatibility of the isolated strains of *C. parasitica* was investigated as well.

*****Corresponding Author:** Gulnara Badridze ✉ gbadridze@yahoo.com

Introduction

Wood of excellent quality and high nutritional value fruits rise the chestnut plant - *Castanea sativa* Mill. in range of popular and economicaly significant trees in the world. (Heiniger and Rigling, 1994). Though, the plant is under the danger of extinction all over the world. The reason of it is so called "chestnut blight" disease caused by the plant pathogenic fungus from ascomycetes *Cryphonectria parasitica* (Murrill) Barr (syn. *Endothia parasitica* (Murrill)) (Anagnostakis, 1994; Rigling and Prospero, 2018).

It is more than half of a century the world scientists try efforts to fight the chestnut blight but in vain. Healing of sick trees with hypovirulent strains is regarded to be the most effective measure against *C. parasitica* today (Robin and Heiniger 2001; Puia *et al.*, 2012). As it has established, hypovirulence is stipulated by the existence of *Cryphonectria hypovirus* (CHV) in the cytoplasm of *C. parasitica*. The genome of the virus is double chain RNA molecule (ds RNA). The virus lacks of capsid, which suppresses its active spreading in the environment. The virus is able "to enter" a new host organism only in case of formation of the hypae anastomoses between two stains of *C. parasitica*, or by means of the host's asexual spores.

Though, wide scale integration of hypovirus under the natural conditions is not always possible because of the vegetative incompatibility of *C. parasitica's* different stains (Anagnostakis, 1977). There exists another method of the biological inspection of chestnut blight: isolation of antagonistic to *C. parasitica* microorganisms from the natural sources and their application against the pathogen. According to literary data among the antagonistic microorganisms of *C. parasitica* are well known genera of microscopic fungi and bacteria (*Trichoderma*, *Penicillium, Bacillus, Streptomyces*) (Wilhelm *et al.,* 1998; Groome *et al.*, 2001; Akilli *et al.,* 2011; Smith, 2013).

Combined application of antagonists and hypovirulent strains of *C. parasitica* appeared especially effective against the disease (Akilli *et al.*, 2011). Those countries which are unable to control chestnut blight by hypovirulent strains, for the purpose to localize the pathogen, apply the easy way of *in situ* struggle against the pathogen – soil compressing or mud packing method (Anagnostakis, 1994; Groome *et al.,* 2001).

The above mentioned botanical disaster of 20th century touched country of Geogia as well. Ten years ago 50% of Georgian chestnut forests were already dead (Prospero *et al.,* 2013). There does not exist any effective fungicide against *C. parasitica*, according to the information of Georgian Ministery of Environment Protection. Cutting of sick trees is the only way of cryphonecrosis localization here. Except the joined scientific project of Swiss and Georgian investigators and attempts of the Turkish scientists of integration of hypovirulent strains in Ajara chestnut forest, practically no active measures have been performed for saving of the unique plant of *Castanea sativa* in Georgia, which is placed in "Georgian red list" and "Red book" (Prospero *et al.,* 2013). Thus, elaboration and testing of the strategy of biological control against cryphonecrosis in Georgian chestnut forests is very urgent task today. Accordingly, the purpose of the presented work was to isolate the virulent strains of *C. parasitica*, spread in chestnut populations of one of the regions of west Georgia (Imereti), and to reveal the effective, antagonistic biological agents against them. The strategy of the fight against chestnut blight, spread in chestnut populations of west Georgian region Imereti, will be elaborated for the first time, and isolation of antagonistic to *C. parasitica* microscopic fungi directly from one of the hot spots of cryphonecrosis of Georgia will be performed for the first time, as well as creation of the collection of antagonists against the pathogen. All this may be regarded as the scientific novelty of the presented work.

Collection of this type may be considered as the material base for management and localization of chestnut blight epidemic in this region. We hope that creation of the antinecrotic bio-preparation and its *in situ* testing on the base of isolated particular antagonist microscopic fungi or their consortia will become possible in future.

Materials and methods

Sampling

The experimental virulent strains of *C. parasitica* were isolated from the sick chestnut trees of Imereti region (west Georgia) chestnut forests, as well as the cultures of microscopic fungi were isolated from the soil samples picked just under the stems of sick trees of above mentioned location.

Pathogen was sampled by means of sterilized lancers and knifes. Samples were taken in depth of 5mm from the infected bark of 8 sick trees, situated in the distance of 10m. For sampling the antagonistic to *C. parasitica* microscopic fungi the soil samples were taken in depth of 15cm in sick chestnut forests (10 avareged samples) directly under the tree truks in the radius of 50cm, from five different places (Fomin, 2001). Samples were placed in sterilized containers and labled with indication of the location of sampling.

The initial inoculates and pure cultures

The initial Petri dishes cultures were obtained after the following procedures have been done: first of all the suspensions of samples were prepared. For this purpose one g of a particular bark-sample was placed in a sterile 250ml conical vessel and was added with 99ml of tap water (the initial suspension). To obtain relatively homogenous suspension of a particular microorganism the diluted samples were placed on shaker, at 30°C, 150rot/min. Almost the same procedures were performed to obtain the suspensions of soil samples, with the difference that 10g of each soil sample was added with 90ml of tap water (the initial suspension). Later the soil suspensions were treated by means of

soil particles dispersion, microorganisms cells desorption and micro colonies separation (into a particular composing cell) methods (Zvyagintsev *et al.*, 1980). As a result the homogenous suspensions were received. The suspensions were cultivated by Waksman's standard method of soils dilution (Waksman, 1916) and soils direct cultivation method (Warcup, 1950). Similar approach was applied while cultivating the suspension of bark samples. Following dilutions were prepared from the initial suspension: 1/10, 1/100, 1/1000, and 1/10000. 0.1ml of the diluted suspensions was inoculated on Petri dishes with the nutritional medium (g/l): 0.5l of wort 7°B, 0.5l of tap water, and 20 g of agar. The pH of the medium was 5.5-6.0, sterilizing regimen – 45min, at 0.7atm.

The initial inoculates were incubated in a thermostat at 28°-30°C during 5days. The pure cultures were isolated after the primary microflora was received. Isolated from soil samples and purified, particular strains were placed in test-tubes, on a sterilized, universal agar nutrient medium and incubated in a thermostat at 28°-30°C during 10days. Mature cultures were stored in a fridge at 4°C, in test-tubes with the universal agar nutrient medium.

Identification of microscopic fungi

From the beginning, till the identification of isolated fungi, the cultures were given the initial letters of the sampling location (E-Eto, D – Darka, Ch- Chala), as well as the number (following numeration). On the first step of identification the cultural-morphological peculiarities of experimental fungi were studied (speed of colonies growth, diameter, size, color, etc.). Later the colonies were observed on Petri dishes under the microscope, and preparations for microscopy were prepared.

Part of the fungal colony, free of the agar of nutrient medium, was cut out with the tip of sterilized loop and placed in water drop for microscopic testing.

The spores spread on the surface of water drop. Ethanol with ratio 1:1, or concentrated acetic acid was added to water drop, as fungal spores and conidia do not adhere to water. The reprint-preparation was prepared as well. For this purpose 10mm in diameter colony was cut out from the agar nutrient medium and placed on the preparation glass with the fungal colony upward. It was accurately and tightly covered with sterile cover glass.

Later the cover glass was placed on the preparation glass, which was previously dropped with water or methylene blue. The prepared sample was investigated by the dry optical system.

Screening of the microscopic fungi

Particular culture of microscopic fungi, isolated from soils were inoculated in the same Petri dish, together with virulent strain of *C. parasitica*, on the universal nutrient medium, for the purpose to reveal antagonistic to chestnut blight microscopic fungi. The extent of inhibition of the growth of parasitic fungus indicated to the antagonistic activity of the neighbor, which was evaluated according to our 5 scale system.

Determination of the vegetative compatibility of C. parasitica virulent strains

The vegetative compatibility of the *C. parasitica* virulent strains (vc-types) was determined on Petri dishes as well. Preliminary sterilized agar nutrient medium with potato and dextrose was inoculated side by side with particular virulent strains. Merging of different colonies, as well as formation of hyphae anastosomosis between virulent strains served as indication to their compatibility.

Results and discussion

The visual evaluation of dissemination and extent of cryphonecrosis in villages (Darka, Eto and Chala) of Imereti region (west Georgia) was the initial step of experiment. On the base of these observations the sampling individuals were selected. 23 strains of *C. parasitica* were isolated, purified and identified from the experimental bark samples. Among them 9 strains were from v. Eto chestnut forest, 10 – from v. Darka forest, and 4 strains – from v. Chala mixed deciduous forest (Fig. 1, 2). Accordingly the collection of *C. parsitica* was created. Guide of microscopic fungi was used for the identification of pathogenic fungus (F.M.Dugan, 2006; Malloch, *et al*. 1981).

Fig. 1. The initial inoculates of the parasitic fungus *C. parasitica*, isolated from the infected chestnut trees.

Fig. 2. The pure, identified cultures of *C. Parasitica.*

From literary data it is known that the plant pathogen fungus – *C. parasitica* may be infected by the dsRNA hypovirus (CHV-*Cryphonectria hypovirus*), which deprives the pathogen of virulence and it becomes hypovirulent (Milgroom and Cortesi, 2004; MacDonald and Double, 2004). The virulent strains of *C. parasitica* differ from the hypovirulent ones phenotipically as well. On agar medium with potato and dextrose (PDA) they form yellow mycelia, with abundant spores. The pigmentation and sporulation of virus-infected *C. parasitica* is significantly low, compared to virulent strains. On PDA they form white colonies. There has not been revealed any clear hypovirulent strain of *C. parasitica* among the isolated cultures. Though, in particular cases morphotypically white, similar to hypovirulent strains, colonies were observed, which need further molecular investigation for identification. After the collection of *C. parasitica* strains was created, the vegetative compatibility between the strains was studied. Six vc types were revealed, that indicates to high genetic diversity of the parasitic fungus of Imereti chestnut forests. The strains of *C. parasitica* of one particular chestnut population appeared to be incompatible with strains isolated from other chestnut populations as well.
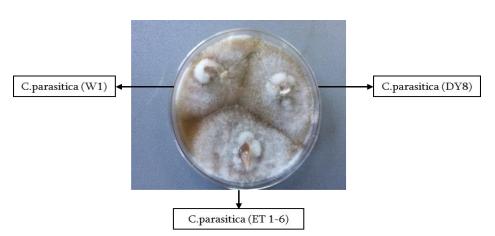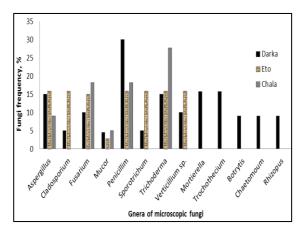
From the infected chestnut populations of Darka, Eto and Chala three incompatible groups of *C. parasitica* have been revealed (Fig. 3).

Fig. 3. Three incompatible groups of *C. parasitica* from the infected chestnut populations of Darka, Eto and Chala.

Since the principle goal of our investigations was to reveal the antagonistic to *C. parasitica* strains of microscopic fungi under natural conditions, on the next step of our study 50 strains of antagonists were isolated and purified from surrounding soils of chestnut trees. Among them 20 strains were from Darkha soils, 19 – from Eto soils, and 11 strains – from Chala chestnut forest soils (Fig.4). After the pure cultures were received, their identification was important. At first the big taxonomic units (class, order) were determined, according to structural peculiarities of reproductive organs and spores (Arx, 1970; Kreisel and Fisher, 1969). For the identification on genus level were used guides after Pidoplichko and Milko (1971), Bilaiy and Koval (1988), Litvinov (1967), Malloch *et al*. (1981), and Fungal Planet (2014).
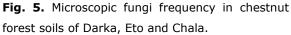
Identified microscopic fungi belonged to genera: Mortierella, Verticillium, Fusarium, Trichoderma, Trichothecium, Sporotrichum, Helminosporium, Botritis, Cladosporium, Aspergillus, Penicillium, Mucor, Chaetomuim, Rhizopus.

Fig. 4. The first inoculates of the microflora isolated from chestnut forest soils.

Fungi Frequency in chestnut forest soils is demonstrated on Fig. 5. The dominating genera of microscopic fingi were revealed in soils of chestnut forests according to data on fungi frequency. Our experimental results are in accordance with literary data about dominance of genera *Penicillium* and *Trichoderma* in forest brown soils (Akilli *et al*., 2011; Groome *et al*., 2001; Smith, 2019). By frequency they are followed by genera *Aspergillus* and *Fusarium.* Quantitatively other genera of microscopic fungi were presented in comparatively low amount.

Fig. 5. Microscopic fungi frequency in chestnut forest soils of Darka, Eto and Chala.

The identification of the microflora of experimental soils has revealed a significant regularity: among those 50 strains of microscopic fungi, which were isolated from tested soils *C. parasitica* was absent. This may be regarded as a confirmation of the antagonistic activity of the "aboriginal" microflora. The next step of experimental work aimed to test the antagonistic effect of a particular strain of isolated micromycetes towards *C. parasitica*. As it was supposed, the representatives of genera *Penicillium* and *Trichoderma* (Fig. 6, a) revealed high antagonistic effect towards the plant pathogen (Akilli *et al.*, 2011). It must be mentioned that new antagonistic to *C. parasitica* genus - *Aspergillus* was revealed in our experiments. No literary data exist on possibility of inhibition of the plant pathogen growth by this fungus. Two species of *Aspergillus* isolated in our experiments - *A. Niger* and *A. Flavus* totally inhibited growth and development of *C. parasitica* strains (Fig. 6, b, c). During observations it was established that the antagonistic effect of micromycetes did not depend on a particular location and was equal towards different strains of *C. parasitica* (Table 1).

Table 1. Screening of antagonistic to *C. parasitica* micromycetes

No	Strains of soil micromycetes	*C. parasitica* ET-1-9	*C. parasitica* W-1-4	*C. parasitica* DY-1-10
1.	*Aspergillus flavus*-DY2	++++	++++	++++
2.	*Aspergillus flavus*-DY3	+++++	+++++	+++++
3.	*Aspergillus flavus*-ET2	+++++	+++++	+++++
4.	*Aspergillus flavus*-ET3	+++++	+++++	++++
5.	*Aspergillus niger* -W1	+++	+++	+++
6.	*Aspergillus niger*-DY1	+++++	++++	++++
7.	*Aspergillus niger*-ET1	+++++	+++++	+++++
8.	*Botritis* sp.-W2	-	-	-
9.	*Chaetomuim* sp.-W3	-	-	-
10.	*Cladosporium* sp.-DY4	-	-	-
11.	*Fusarium* sp.-DY 17	++	++	++
12.	*Fusarium* sp.-DY 18	+++	+++	+++
13.	*Fusarium* sp.-ET17	+++	+++	+++
14.	*Fusarium* sp.-ET18	+++	+++	+++
15.	*Fusarium* sp.-W10	-	-	-
16.	*Fusarium* sp.-W11	-	-	-
17.	*Helminosporium* sp.-E19	-	-	-
18.	*Mortierella* sp.-ET5	-	-	-
19.	*Mucor* sp.-DY5	-	-	-
20.	*Mucor* sp.-DY6	+	+	+
21.	*Mucor* sp.-ET 4	+	+	+
22.	*Penicillium* sp.- ET7	+++	+++	+++
23.	*Penicillium* sp.-DY10	+++++	+++++	+++++
24.	*Penicillium* sp.-DY11	++	++	++
25.	*Penicillium* sp.-DY12	++	++	++
26.	*Penicillium* sp.-DY7	++	++	++
27.	*Penicillium* sp.-DY8	++	++	++
28.	*Penicillium* sp.-DY9	+++++	+++++	+++++
29.	*Penicillium* sp.-ET6	-	-	+
30.	*Penicillium* sp.-ET8	++++	++++	++++
31.	*Penicillium* sp.-ET9	+++++	+++++	+++++
32.	*Penicillium* sp.-W4	+	+	+
33.	*Penicillium* sp.-W5	+++++	+++++	+++++
34.	*Rhizopus* sp.-W6	-	-	-
35.	*Sporotrichum*sp.-DY13	-	-	-
36.	*Trichoderma viride* -W7	+++++	+++++	+++++
37.	*Trichoderma viride* -W8	+++++	+++++	+++++
38.	*Trichoderma album* -ET13	-	-	-
39.	*Trichoderma* sp.-ET14	-	-	-
40.	*Trichoderma* sp.-ET15	-	-	-
41.	*Trichoderma viride* -DY14	+++++	+++++	+++++

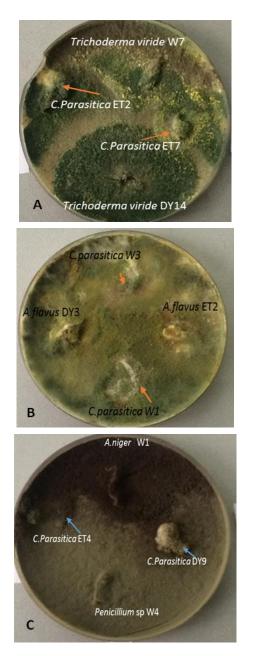| 42.	*Trichoderma viride* -DY15	+++++	+++++	+++++
43.	*Trichoderma viride* -ET11	++	++	++
44.	*Trichoderma viride* -ET12	++	++	++
45.	*Trichoderma viride*-DY16	++	+	++
46.	*Trichoderma viride*-ET10	+++++	+++++	+++++
47.	*Trichoderma viride*-W9	+++	+++	+++
48.	*Trichothecium*-ET16	-	-	-
49.	*Verticillium* sp.-DY19	++	++	++
50.	*Verticillium* sp-.DY20	++	++	++

Fig. 6, a. representatives of genera *Penicillium* and *Trichoderma* revealed high antagonistic effect towards the plant pathogen. **b, c.** Two species of *Aspergillus - A. Niger* and *A. Flavus* totally inhibited growth and development of *C. parasitica* strains.

Collection of the antagonistic to *C. parasitica* microscopic fungi, created on the base of screening, may be concerned as the material base for management and localization of the chestnut blight epidemy. Creation of the antinecrotic preparation on the base of a particular antagonistic strain or their consortium and its *in situ* examination in future seems possible.

Summarizing, the following significant results may be distinguished:

1. 23 strains of *Cryphonectria parsitica* (Murrill) Barr were isolated, purified and identified from the cryphonecrosis-sick chestnut populations of three Imeretian (west Georgia) villages: Darka – 10 strains , Eto -9 strains, and Chala – 4 strains. The collection of the parasitic fungus has been created and the vegetative compatibility of the strains was investigated.

2. Six vegetatively incompatible (vc) types of *C. parasitica* were revealed.

3. 50 strains of microscopic fungi were isolated and purified from soils of infected chestnut forests for the purpose to reveal biological antagonists of *C. parasitica*. Among them 20 strains were from Darka soils, 19 – from Eto soils, and 11 strains- from Chala chestnut forest soils. Collection of mycromycetes was created.

4. The identified mycromycetes belonged to following genera: *Mortierella, Verticillium, Fusarium, Trichoderma, Trichothecium, Sporotrichum, Helminosporium, Botritis, Cladosporium, Aspergillus, Penicillium, Mucor, Rhizopus* and *Chaetomuim*.

5. Frequency of mycroscopic fungi in soils of infected chestnut forests cleared the dominating genera of the first (*Penicillium* and *Trichoderma*)

and second order (*Aspergillus* and *Fusarium*). Other genera were presented in small amount.

6. Investigation of the antagonistic effect of soil "aboriginal" microflora has revealed strong biological antagonists of the plant pathogen among particular strains of *Penicillium* and *Trichoderma*.

7. New, antagonistic to *C. parasitica* genus – *Aspergillus* has been revealed. Two species of this genus *A. niger* and A*. flavus* completely inhibited growth and development of the virulent strains of the pathogen*.*

References

Akilli S, Katircioğlu YZ, Maden S. 2009. Vegetative compatibility types of Cryphonectria parasitica, causal agent of chestnut blight, in the Black Sea region of Turkey. Forest Pathology **39(**6**),** 390-396.

Akilli S, Katircioğlu YZ, Maden S. 2011. Biological control of chestnut canker caused by *Cryphonectria parasitica*, by antagonistic organisms and hypovirulent isolates. Turkish Journal of Agriculture and Forestry **35(5),** 515-523.

Anagnostakis SL. 1977. Vegetative incompatibility in Endothia parasitica. Experimental Mycology **1(4),** 306-316.

Anagnostakis SL. 1994. Protecting chestnut trees from blight. Northern Nut Growers Association (USA).

Arx, von JA. 1970. The genera of fungi sporulating in pure culture. Gramer Lehre, 1-288.

Bilaiy VI, Koval EZ. 1988. Aspergills. Kiev, Naukova Doumka (in Russian).

Crous PW, Shivas RG, Quaedvlieg W, Van der Bank M, Zhang Y, Summerell BA, Guarro J, Wingfield MJ, Wood AR, Alfenas AC, Braun U. 2014. Fungal Planet.

Dugan FM. 2006. The Identification of Fungi. An Illustrated Introduction with Keys, Glossary and Guide to Literature. APS Press, St. Paul, MN. USA. 176.

Fomin GS, Fomin AG. 2001. Soil. Monitoring on Quality and Ecologic Safety in Accordance with International Standards. Moscow. VNII standard (in Russian).

Groome PC, Tattar TA, Mount MS. 2001. Bacteria found on American chestnut bark and their potential in biocontrol of chestnut blight. Arboricultural Journal **25(3),** 221-234.

Heiniger U, Rigling D. 1994. Biological control of chestnut blight in Europe. Annual review of phytopathology **32(1),** 581-599.

Kreisel H, Fisher G. 1969. Grundzuge eines haturlichen systems der pilze. Jena.

Litvinov A. 1967. Guide of microscopic soil fungi. Leningrad (in Russian).

MacDonald WL, Double ML. 2004. Hypovirulence: use and limitations as a chestnut blight biological control. In: Restoration of American chestnut to forest lands. Proceedings of a Conference and Workshop 7-95.

Malloch D. 1981. Moulds, their isolation, cultivation, and identification. University of Toronto Press.

Milgroom MG, Cortesi P. 2004. Biological control of chestnut blight with hypovirulence: a critical analysis. Annu. Rev. Phytopathol **42,** 311-338.

Pidoplichko NM, Mylko AA. 1971. Atlas of Mucosal Fungi. Kiev, Naukova Dumka (in Russian).

Prospero S, Lutz A, Tavadze B, Supatashvili A, Rigling D. 2013. Discovery of a new gene pool and a high genetic diversity of the chestnut blight fungus *Cryphonectria parasitica* in Caucasian Georgia. Infection, Genetics and Evolution **20,** 131-139.

Puia CE, Grogorescu DA, Miclea RV. 2012. The Morphology and the Biological Control of Cryphonectria parasitica. Bulletin of the University of Agricultural Sciences & Veterinary Medicine Cluj-Napoca. Agriculture **69(1).**

Rigling D, Prospero S. 2018. Cryphonectria parasitica, the causal agent of chestnut blight: Invasion history, population biology and disease control. Molecular plant pathology **19(1),** 7-20.

Robin C, Heiniger U. 2001. Chestnut blight in Europe: diversity of Cryphonectria parasitica, hypovirulence and biocontrol. Forest Snow and Landscape Research **76(3),** 361-367.

Smith AR. 2013. Biological Control of Cryphonectria Parasitica with Streptomyces and an Analysis of Vegetative Compatibility Diversity of Cryphonectria Parasitica in Wisconsin, USA. Doctoral dissertation, University of Wisconsin--La Crosse.

Waksman SA, Curtis RE. 1916. The actinomyces of the soil. Soil Science **1(2),** 99-134.

Warcup JH. 1950. The soil-plate method for isolation of fungi from soil. Nature **166,** 117.-118.

Wilhelm E, Arthofer W, Schafleitner R, Krebs B. 1998. Bacillus subtilis an endophyte of chestnut (Castanea sativa) as antagonist against chestnut blight (Cryphonectria parasitica). Plant cell, tissue and organ culture **52(1-2),** 105-108.

Zviagintsev NDG. 1980. Methods of Soil Microbiology and Biochemistry. Moscow University, 12-14. (in Russian).